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Neurodevelopmental polybrominated diphenyl ether exposures

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by

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Acknowledgements

Chapter 2

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Chapter 3

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Chapter 4

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- **Poston RG,** Murphy L, Rejepova A, Ghaninjad-Esfahani M, Segales J, Mulligan K, Saha RN; Specific ortho-hydroxylated brominated ethers inhibit neuronal MEK-ERK signaling and disrupt neurodevelopmental processes, *J Biol Chem*; (2019) **(In revision)**
- **Poston RG,** Saha RN; Epigenetic effects of polybrominated diphenyl ethers on human health, *Int. J. Environ. Res. Public Health*; 16, 1–13 (2019) **(Invited review)**
- **Poston RG**, Dunn CJ, Sarkar P, Saha RN; Persistent 6-OH-BDE-47 exposure impairs functional neuronal maturation and alters expression of neurodevelopmentally-relevant chromatin remodelers, *Environmental Epigenetics*; 4:1 (2018) - **(*)**
- Tyssowski KM, DeStefino NR, Cho JH, Dunn CJ, **Poston RG**, Carty C, Jones RD, Chang SM, Romeo P, Wurzelmann MK, Ward JM, Andermann ML, Saha RN, Dudek SM, Gray JM; Different neuronal activity patterns induce different gene expression programs, *Neuron*; 98:1-17 (2018) **- (*)**
- **Poston RG**, Masthay MB; Characterization of the role of oxygen in the photodegradation of short-chain diphenylpolyenes and β-carotene, (2014) (Non-peer reviewed undergraduate thesis)

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- Society for Neuroscience, Neuroscience 2019 Meeting– poster presentation: *Neurodevelopmental disruptions by PBDES are mediated in part by inhibition of neuronal MEK-ERK signaling*. Chicago, Illinois (October 2019)
- Society for Neuroscience, Neuroscience 2019 Meeting– poster presentation: *Two functionally distinct signaling cascades are required to efficiently couple neuronal activity with immediate early gene transcription*. Chicago, Illinois (October 2019)
- NIEHS Epigenetics, Stem Cells, and Environmental Health Symposium– poster presentation: *Persistent 6-OH-BDE-47 exposure impairs functional neuronal maturation and alters expression of neurodevelopmentally-relevant chromatin remodelers*. NIEHS. Durham, North Carolina (June 2017)

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Abstract

Neurodevelopmental polybrominated diphenyl ether exposures

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Humans around the world are exposed to many potentially toxic compounds of both natural and anthropogenic origin. Industrial chemicals represent a large fraction of such compounds, some of which are now considered widespread environmental pollutants. One such class of compounds is polybrominated diphenyl ethers (PBDEs), a widely prevalent persistent organic pollutant spread largely by anthropogenic production and use of these chemicals as flame retardants in consumer products. The lipophilic nature of PBDEs, which are small halogenated organic molecules, allows them to accumulate in lipid-rich bodily tissues, including the brain. Due to the exposure kinetics of these compounds (detailed in Chapter 4 of this dissertation), cortical astrocytes and neurons are likely exposed in a region-specific manner. Concerningly, there is now a growing body of evidence epidemiologically linking PBDE exposure levels and incidences of behavioral deficits related to neurodevelopmental disorders (NDDs) in human children. NDDs include intellectual disability, attention deficit disorders, and autism spectrum disorders that are estimated to affect 15-20% of children in the United States. This association is supported by evidence from non-human animal studies that has established that exposures to several PBDEs affect learning. In an effort to understand how exposures to these compounds is related to observed behavioral effects, several major mechanisms have been identified, including: disruption of calcium homeostasis, interference with hormonal signaling, cellular toxicity resulting from mitochondrial disruption and the production of reactive oxygen species, and a more recently emerging focus, disruption of epigenetic mechanisms. Despite much progress, there is not a clear understanding of how molecular and cellular effects of PBDE exposure are related to observed impacts on behavior and learning. In this dissertation, I first tested the hypothesis that the effects of neurodevelopmental PBDE exposures intersect with epigenetic regulators in embryonic cortical neurons, leading to the development and testing of a mechanistic hypothesis of intracellular signaling disruption caused by ortho-hydroxylated PBDE metabolites. I close by detailing a model of neurodevelopmental PBDE exposures that I combine with novel data generated here to generate hypotheses for future study. This body of work aims to be of value in continuing to build an understanding of the effects of PBDE exposures in the developing nervous system, how these exposures are related to observed behavioral deficits in animals, and generating insight regarding the interplay between genetic and environmental risk factors in neurodevelopmental disorders.

Chapter 1: Introduction – Polybrominated diphenyl ethers, epigenetic regulation, and neurodevelopmental disorders

Across the world, humans face exposure to a vast number of industrial chemicals, whose potential for negatively impacting human health has long been a concern (Smyth 1946; Gehrmann 1946; Henry 1946). In early 2018, the United States Environmental Protection Agency (EPA) reported 30,972 active chemicals in industry out of a total of 86,071 registered in the agency's Toxic Substances Control Act (TSCA) Chemical Substance Inventory. The European Chemicals Agency's (ECHA) most recently updated figure from their relatively new Regulation for Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) initiative reports 21,403 unique substances. China has also established a program recently—similar to Europe's REACH regulations—that mandates new updating of China's chemical inventory, the Inventory of Existing Chemical Substances (IECSC), which lists 45,612 substances as of 2013. As world governments attempt to define what chemicals have been produced and are in use, efficient methods to identify and evaluate compounds for safety screening are still being debated and formed. Progress is slow, with few chemicals actually being heavily regulated. In the US, the history of chemical regulation is long and convoluted, and is well reviewed elsewhere (Krimsky 2017). Presently, the EPA is in the midst of a three-tiered evaluation program designed to assess the safety of existing chemicals, with only the most dangerous chemicals likely to ever reach the eventual 'Risk Management' phase. It is questionable whether this type of approach is practical at all, yet meaningful change may not come soon, as it is unlikely that the country will shift the burden of proof regarding chemical safety from regulatory agencies to manufacturers (as with Europe's REACH program). In the meantime, the vast volume and diversity of industrial chemicals we expose ourselves to continues to pose a potentially serious risk to human health. There are numerous avenues by which hazardous compounds may impact human health, perhaps the most widely recognized of which are potential for carcinogenicity, adverse effects on reproductive health, and disruption of hormonal signaling. Another exceedingly concerning endpoint for human health is nervous system toxicity, particularly during development of the brain. The developing brain is an especially vulnerable target due to the complex nature of its formation and refinement that spans prenatal and years of postnatal development. As such, neurodevelopmental toxicity induced by chemical exposures has been heavily studied (Grandjean and Landrigan 2014), but much remains unclear. In the body of work presented here, I will focus on a class of industrial chemicals that has been under heavy scrutiny for suspected neurodevelopmental toxicity: polybrominated diphenyl ethers (PBDEs).

PBDEs are a group of environmentally persistent chemicals that were first synthesized in 1871 by the German chemist Wilhelm Hoffmeister (Hofmeister

1871). In 1960, they were patented for use as flame retardants and subsequently have been widely used in household consumer products. Due to their environmental stability and propensity for bioaccumulation, PBDEs have since globally accumulated in the environment and in our bodies. Intriguingly, PBDEs enter the environment from natural sources in addition to industrial production. The compounds were first described in the biomedical literature as early as the 1960s—around the very beginning of their heavy anthropogenic production and use—when they were isolated from Australian marine sponges (*Dysidea sp.*) and found to have antimicrobial properties (Sharma and Burkholder 1967; Burkholder and Sharma 1969; Sharma *et al.* 1970). They have also been isolated from various red algae (Malmvärn *et al.* 2008; Malmvärn *et al.* 2005). Recently, in the case of sponges, it was demonstrated that PBDEs are actually produced by symbiotic cyanobacteria and are theorized to confer some level of microbial resistance to the host sponges, although the mechanism(s) by which the compounds are toxic to other organisms remains unknown (Agarwal *et al.* 2017). It is interesting to note, however, that these compounds are excreted by the cyanobacteria and subsequently accumulate in high concentrations, crystalizing in the sponge ectosomal tissues. This is perhaps how sponges avoid the compounds' toxic effects and how they may be a defense mechanism against potential eukaryotic predators such as fish in addition to other prokaryotes (Unson *et al.* 1994). In the context of human health, it is unfortunate that such a class of compounds, whose natural production was likely evolutionarily driven by their toxicity, ended up becoming a flame retardant of choice for consumer products. Understanding the natural origins of PBDEs may also inform our investigation of their biological effects in humans, which is of pressing importance given another unfortunate aspect of PBDE biology—the growing evidence for their epidemiological association with neurodevelopmental disorders (NDDs). In this chapter, I will discuss known biological mechanisms affected by PBDEs, focusing on epigenetic impairments and the impacts these disruptions may have on human health, especially in the context of neurodevelopmental disorders.

1.1 Relation of PBDEs to Human Health

1.1.1 Human Exposure to PBDEs and Effects on Human Health

A major effort has been devoted to evaluating the potential risk of PBDE exposure to human health. For the sake of brevity, here I will highlight some of the major points while pointing to relevant reviews and meta-analyses of the vast number of studies that have been published on the topic. Monitoring of environmental and human levels of PBDEs in the 1990s led to a rising concern that they may pose a serious human health risk, a concern which became widely recognized by the early 2000s (Eriksson *et al.* 2001). At the time, the extent the of compounds' toxicity was unclear despite increasing amounts of toxicological evidence. Following much attention in the time since, it is now clear that exposure to PBDEs is a very real concern for humans around the world as the compounds are environmentally stable and lipophilic, and thus tend to

bioaccumulate and also collect in households, primarily in dust. These routes of accumulation enable the most common modes of human exposure—primarily through ingestion and inhalation of dust (Frederiksen *et al.* 2009; Bramwell *et al.* 2016) and dietary intake, predominantly from seafood and dairy products (Linares *et al.* 2015). It is also concerning and of interest that infants and toddlers tend to have higher body burdens compared to adults when considering potential developmental toxicity (U.S. Environmental Protection Agency (EPA) 2010). This is thought to be caused by younger children having higher rates of intake from dust and household products, as well as by additional exposure to PBDEs through breastmilk.

Due to the persistent, widespread, and sometimes heavy exposure levels observed, much attention has been given to the roles of PBDEs in several major aspects of health: carcinogenicity, reproductive health, and disruption of hormonal signaling (Costa *et al.* 2008; Linares *et al.* 2015; Gorini *et al.* 2018). In addition to these concerning PBDE-related effects on human health, another serious worry is their neurotoxicity and potential roles in the etiology of neurodevelopmental disorders (NDDs). NDDs are a heterogeneous class of developmental disorders of the nervous system including autism spectrum disorders (ASD), attention deficit/hyperactivity disorder (ADHD), intellectual disability (ID), and schizophrenia. It is estimated that up to 15% of children in the United States are affected by such disorders (U.S. Environmental Protection Agency (EPA) 2015). Despite their growing prevalence, there remains much that is unknown regarding the etiologies of the disorders. With the continuing emergence of sequencing technologies, much effort and hope has been put into disentangling the complex origin of NDDs at the level of genetic mutation/variation, largely through whole-genome sequencing and whole-exome sequencing studies of affected individuals and their families (Jeste and Geschwind 2014; Cardoso *et al.* 2019). This effort has led to an understanding that complex networks of genetic mutations underlie NDDs, rather than most risk being account for by highly penetrant mutations as with Mendelian diseases (Parikshak *et al.* 2015; Hu *et al.* 2014). In addition, it has come to be increasingly appreciated that additional factors such as environmental exposures must also be accounted for to understand the complex risk interactions giving rise to NDDs (De Felice *et al.* 2015).

A substantial amount of work has been done, surveying the potential association of PBDE exposures with representative behavioral deficits in humans, as well as in other animal models. Animals exposed to various PBDEs during prenatal and/or postnatal periods exhibit long-lasting behavioral abnormalities, including deficiencies in motor activity and cognitive functions (Eriksson *et al.* 2001; Gee and Moser 2008; Branchi *et al.* 2002; Dufault *et al.* 2005). Along similar lines, epidemiological studies in human populations have reported significant associations of maternal neonatal PBDE exposure with deficits in motor behavior, Intelligence Quotient (IQ) ratings (showing intellectual

disability), and attention and cognitive functions in children (Herbstman and Mall 2015; Herbstman *et al.* 2010; Eskenazi *et al.* 2013; Chevrier *et al.* 2016; Cowell *et al.* 2015; Chen *et al.* 2014). Recently, an expert panel identified a 70-100% probability that exposure to PBDEs contributes to IQ loss and intellectual disability that cost the EU public an estimated €9.59 billion (Bellanger *et al.* 2015). Among other relevant findings, a significant positive correlation has been established between concentration of PBDEs in postpartum breast milk and increased externalizing behavioral problems (Hoffman *et al.* 2012) (reminiscent of ADHD-like behavior in older children), as well as between PBDE concentration in peripheral or cord blood and increased risk of attention symptoms and poorer social competency scores (Gascon *et al.* 2011). Summarizing these and other studies, several large-scale and systematic reviews have recently been conducted, both of evidence from human (Vuong *et al.* 2018; Lam *et al.* 2017; National Academies of Sciences and Medicine 2017) and animal studies (Dorman *et al.* 2018). Briefly, they conclude that PBDE exposures highly correlate with externalizing behaviors and IQ in children, while BDE-47/99/209 were specifically concluded to affect learning in animal studies. There is also concern for the relationship between environmental toxins such as PBDEs and autism spectrum disorders, although the relationship is less clear, especially in human studies (Ye *et al.* 2017). Given the established and suspected connections between PBDE exposures and intelligence and behavioral deficits, as well as other aspects of human health, it is imperative to strive for a mechanistic understanding of PBDE toxicity at the molecular and cellular level.

1.2 Biological mechanisms of PBDE toxicity

1.2.1 Known Mechanisms

Since the rise of concern regarding PBDE toxicity, several major impacted biological mechanisms have been identified and investigated. These and other less explored effects of PBDEs have been recently reviewed (Costa et al. 2014). Briefly, major identified points of toxicity are: (1) disruption of calcium signaling—dating back to one of the earliest functional studies of PBDEs (Bussau et al. 1993) and more recently demonstrated in multiple cell types including human neuronal precursors (Dingemans et al. 2008; Gassmann et al. 2014); (2) interference with endocrine signaling—thought to be enabled by structural similarity to various hormones (Sueyoshi et al. 2014; Pacyniak et al. 2007; Wahl et al. 2010; Dingemans et al. 2011); (3) toxicity produced by mitochondrial disruptions including uncoupling of oxidative phosphorylation (van Boxtel et al. 2008) and subsequent elevated production of reactive oxygen species (ROS) which can lead to DNA damage and apoptosis. Many of the details of these known effects remain unresolved and other unidentified mechanisms are likely also affected. Additionally, much of the information on these established effects is derived from experimental exposures to individual PBDEs, while there are 209 congeners that humans are theoretically exposed to in mixtures, and these

compounds can be further processed to produce various metabolites. Such metabolism, thought to be initially endogenously mediated in humans by cytochrome P450 enzymes (Feo et al. 2013a; Erratico et al. 2013; Fu et al. 2016) and inherent in some other natural contexts (Agarwal et al. 2014; Agarwal et al. 2017; Malmvärn et al. 2005; Malmvärn et al. 2008), leads to the production of hydroxylated and methoxylated forms that have received less attention thus far. It has additionally been shown that these oxygenated metabolites can be further modified, producing glucuronidated and sulfated forms (Erratico et al. 2015; Cisneros et al. 2019), for which there are no data available in terms of biological effects. Addressing this lack of information regarding PBDE metabolites is important given that human exposure studies suggest that several OH-PBDEs are present in serum at concentrations similar to, and sometimes higher than,

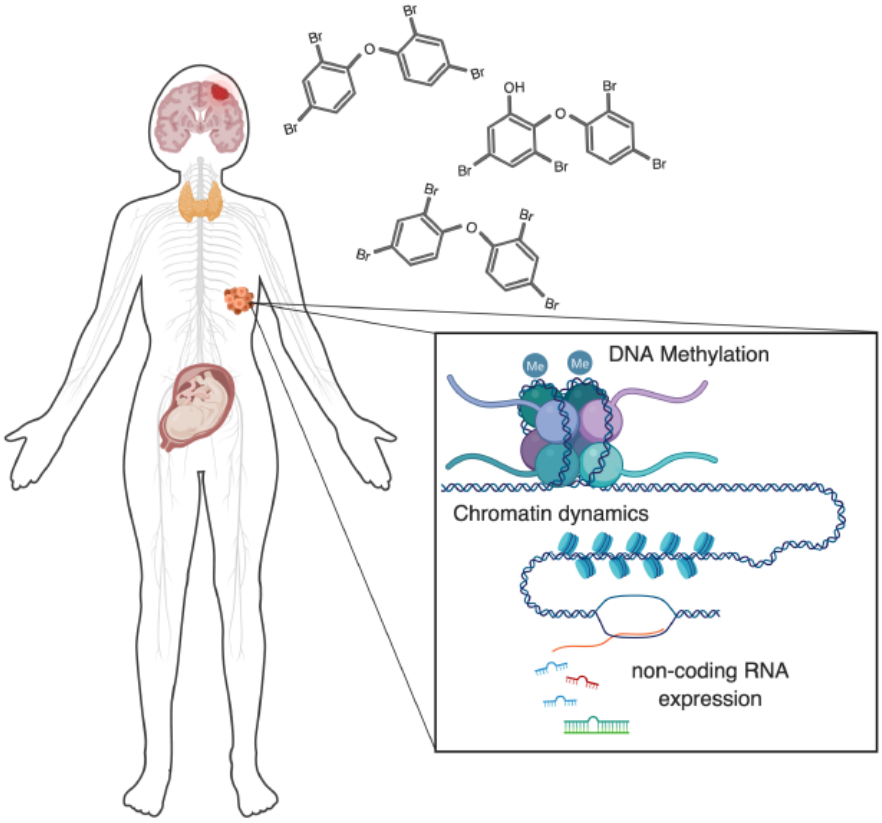

Figure 1-1. PBDE exposures affect epigenetic regulatory mechanisms at multiple levels, across multiple biological systems. There are several major aspects of human health that are of concern regarding PBDE toxicity that now have evidence for an involvement of dysregulated epigenetic regulation. These include: nervous system toxicity, disruption of thyroid hormone signaling, effects on the reproductive system (primarily on the placenta and testes), and oncogenic potential. Epigenetic components are known to be disrupted in each of these systems. In this review, we discuss these epigenetic regulators, their known modes of disruption by PBDEs, and the relationship of these disruptions to human health.

those of parent PBDEs (Qiu et al. 2009; Athanasiadou et al. 2008) and that OH-PBDEs appear to be more capable of competitively binding the thyroid hormone transporter transthyretin, potentially mediating increased endocrine-disruption (Meerts et al. 2000; Hamers et al. 2008; Hamers et al. 2006; Cao et al. 2010; Ren and Guo 2012). Other confounding factors include that past studies have covered a wide range of concentrations, many likely higher than environmentally relevant exposures, and much of this work has been conducted in cell culture, requiring further in vivo confirmation. Thus, while an extensive amount of work has been done, there is much that may yet be uncovered regarding biological mechanisms of PBDE toxicity.

A growing approach towards understanding PBDE toxicity has focused on PBDE-induced disruption of epigenetic regulation (**Figure 1**). Such mechanisms are interesting as potential PBDE targets as they would constitute a gene-environment interface for cellular disruption, wherein the effects of exposure to environmental factors interact with genomic elements, perhaps even with known genetic risk factors for human disease like NDDs (Tran and Miyake 2017). Such interactions could contribute towards explaining the elusive environmental portion of risk thought to contribute to NDD etiology. This notion is supported by the growing appreciation for the role of epigenetic mechanisms in neurodevelopment and cognition (Lasalle *et al.* 2013; Marshall and Bredy 2016), as well as in diseases of the nervous system, including neurodevelopmental disorders (Millan 2013; Christopher *et al.* 2017). Therefore, it may prove useful to understand the effects of PBDE exposures on epigenetic components, across cell and tissues types but particularly in the brain, in order to build more complete causal models towards explaining observed links to human health complications and behavioral deficits. However, compared to other widely studied mechanisms, relatively little attention has been given to epigenetic effects of PBDEs. Here, I will summarize the findings of studies conducted to date that have observed epigenetic endpoints such as DNA methylation, chromatin characteristics including modifications and remodeling of histones, and other epigenetic mechanisms such as expression of various non-coding RNAs.

1.2.2 Repeatedly Observed Disruption of DNA Methylation

One of the most commonly recognized epigenetic mechanisms is DNA methylation. Methylation commonly occurs at cytosine nucleotides positioned before a guanosine (CpG dinucleotides), resulting in a 5-methylcytosine. Given the extensive impact methylation has on transcriptional regulation (Bird 2002), it comes as little surprise that its disruption has potential impacts on human health (Bergman and Cedar 2013). An interesting example is folate deficiency's implication in disrupted methylation during pre-natal development, although the relationship is incompletely understood (Crider et al. 2012). The relationship between PBDE exposure and DNA methylation is similarly incompletely understood. Although most studies report some correlation, they do not have a

clear consensus, especially in human samples, while in vitro studies more consistently report negative correlations (see references below).

Studies have assessed PBDE-exposure-dependent changes in global DNA methylation at various representative regions or at specific loci (gene promoters). Two of the most prominent examples of representative methylated regions include ALU elements and LINE1, which are common transposable repeats that can have adverse cellular impacts when de-repressed due to hypomethylation (Eden *et al.* 2003). Repetitive elements make up a large portion of the human genome (de Koning *et al.* 2011) and have high CpG frequency, contributing heavily to the global amount of DNA methylation and thereby serving as a reasonable global estimate.

One of the earliest studies on the effects of PBDE exposure on global DNA methylation in humans found a negative relationship between measured BDE-47 levels and ALU %5mC in blood samples of healthy Korean adults, while not finding significant relationships for BDE-99 or LINE1 methylation (Kim *et al.* 2010). Similar studies correlating PBDE levels in blood with methylation have followed. One found an inverse relationship between BDE-47 abundance and TNFα promoter methylation in cord blood samples from mother–infant pairs of the Boston Birth Cohort (Dao *et al.* 2015). Another reports a more complex finding in newborn cord blood samples from the CHAMACOS study, wherein significant changes in LINE1 methylation were found when considering co-exposure to DDT, DDE, and PBDEs (the direction of change depended on level of DDE or DDT co-exposure) (Huen *et al.* 2014).

Several groups have also examined the relationship between PBDE levels and effects on the placental epigenome. In 2016, two reports were published on effects in human placental samples. In one, the authors made PBDE, PCB, DDE measurements in villous placental tissue samples and found positive associations of PBDE levels with IGF2/H19 imprinting and methylation status (bisulfite conversion and targeted pyrosequencing) and global DNA methylation (assessed by LUMA (luminometric methylation assay)) (Kappil *et al.* 2016). In the other, PBDE levels in umbilical cord blood were measured from eighty human samples and correlated with placental DNA methylation levels in LINE1, NR3C1, and IGF2. BDE-66/153/209 were all found to have significant negative correlations with methylation of some of these loci (Zhao *et al.* 2016). Two very recent reports have also been made utilizing *in vitro* models of the placenta. One group exposed primary villous cytotrophoblasts (CTBs, an *in vitro* model of human placental development) to BDE-47 or BDE-99. They found that BDE-47 alters gene expression in a concentration-dependent manner and produced a low-level global increase in DNA methylation (assessed with HumanMethylation450 beadarray) (Robinson *et al.* 2019). Another group exposed human placental choriocarcinoma cells (BeWo cells) to 1μM BDE-47

and found reduced methylation of some CpG loci of mitochondrial biomarkers (with no differences found for 50 µMexposures) (Shan *et al.* 2019).

In addition to these human studies, PBDE-methylation relationships have also been investigated in model animals—mostly rodents—both *in vitro* and *in vivo*. *In vitro* studies have been conducted in different cell types, but consistently found negative correlations between PBDE exposure and methylation level. In the earliest of these studies, primary hippocampal neurons were exposed to various concentrations of BDE-209 for 24 hours and subsequently, a global decrease in DNA methylation was found by an antibody based 'ELISA-like' assay (Chen *et al.* 2010). Another found decreased global DNA methylation after a 10µM BDE-47 in murine N2A cells (assessed by HPLC and arbitrary primed PCR). This decrease coincided with increased adipocyte differentiation (2.5–25 µM exposures) (Bastos Sales *et al.* 2013). In a related effort to understand how endocrine-disrupting chemicals may be inducing adipocyte differentiation, investigators report that BDE-47 induces demethylation of several sites in the PPARγ promoter (a key adipongenic transcription factor) in COS7 and 3T3-L1 cells using Methylation-Sensitive High-Resolution Melting (MS-HRM) (Kamstra *et al.* 2014). Complementing these *in vitro* findings, *in vivo* studies that perinatally exposed rodents to BDE-47 reported interesting findings from offspring of various ages. These include decreased expression of LINE1 RNA (Suvorov and Takser 2011), decreased methylation of Mt-co2, L1Rn, Bdnf, and Nr3c1 (Byun *et al.* 2015), differentially methylated regions in sperm (Suvorov *et al.* 2018), and global DNA hypomethylation associated with behavioral deficits in both exposed wild-type and MeCP2-deficient female mice (Woods *et al.* 2012). Another study in mice assessing liver carcinoma tissue after DE-71 (a commercial mixture of PBDEs) exposure found little effect on global DNA methylation but reports a gene body methylation decrease in Tbx3 and subsequent mRNA and protein upregulation (Shimbo *et al.* 2017). While not directly assessing DNA methylation, a multigenerational study in zebrafish that exposed F0 animals to a PCB and PBDE mixture found disrupted behavior (hyper/hypoactivity) in F1–F4 larvae, as well as altered c-Fos expression (F1/2) and altered *Dmnt3ba* expression in all generations (Alfonso *et al.* 2019).

To our knowledge, only two reports exist that found no relationship between PBDE exposure and DNA methylation levels of any targets measured in those studies. One found no detectable decrease in methylation at the p53 promoter after 24 hours of exposure to low micromolar doses (1, 5, 10 µmol/L) of BDE-47 in human neuroblastoma cells (SH-SY5Y), although activation of the p53 pathway in general was implicated in observed effects (Zhang *et al.* 2013b). The other found no significant relationship between BDE-47 serum levels and global methylation as assessed by the luminometric methylation assay (LUMA) in samples from an elderly Swedish population. However, significant relationships were established for other persistent organic pollutants including PCBs and the dioxin OCDD (Lind *et al.* 2013). Aside from these reports, the literature suggests

a fairly consistent—but not necessarily linear—relationship between PBDE exposures and DNA methylation levels. It is possible that changes may vary from genomic region to region and may not always manifest an altered phenotype. Also, there is scarce evidence concerning direct cause–effect relationships between methylation changes and behavioral phenotypes. To aid in filling this gap in knowledge, it may prove useful to further refine understanding of the route by which PBDEs affect DNA methylation states—be it primarily by dysregulation of DNA methyltransferase expression, cellular metabolism, intracellular signaling pathways, etc.

1.2.3 Impact on Chromatin—Histone Modifications to Chromatin Remodeling

Other reversible chemical modifications of chromatin include modifications to histone proteins that regulate chromatin structure and instruct remodeling processes, ultimately controlling gene expression (Venkatesh and Workman 2015; Clapier et al. 2017). Studies starting as early as 2003 reported mixed results on PBDE-induced alterations of chromatin by several measures (chromosomal integrity, chromatin density and localization). Exposure of multiple bacterial strains to BDE-99 did not induce mutagenicity or a detectable increase in the number of structural chromosomal aberrations, while exposure to the PCB mixture Aroclor® 1254 did (Evandri et al. 2003). This early study explicitly stated that the possibility of PBDEs acting through epigenetic mechanisms could not be ruled out, which, in retrospect, was prudent foresight. Two subsequent studies have also reported no increase in degraded chromatin, both in sperm—the first in the sperm of mice orally exposed to BDE-209 (Tseng et al. 2006), the other in human samples of 153 men from the greater Montreal area, despite establishing a correlation between BDE-47 levels and decreased sperm concentration (Albert et al. 2018).

However, there have also been a few studies that do report chromatin disruption following PBDE exposure. One study found that 24-hr nanomolar range exposures to several PBDEs (BDE-47/99/153/183/209) induced micronuclei formation during cytokinesis in MCF-7 cells, an indicator of chromosomal damage occurrence preceding cell division (Barber *et al.* 2006). It has also been found that rat pups exposed to a single injection of BDE-153 at post-natal day 10 (PND10) exhibited behavioral dysfunction in a dose- and age-dependent manner one or two months later. Neurons in the CA3 region of the hippocampus of these rats were also found to be undergoing significantly increased rates of apoptosis, with chromatin condensed and localized to the nuclear membrane (Zhang *et al.* 2013a). Most recently, it was reported that BDE-209 exposures reduced hESC differentiation (although total induction was still greater than 90%) and also led to chromosomal copy number variants (CNVs), as well as decreased expression of DNMT1/3A (Du *et al.* 2018).

There is also some evidence specifically for PBDE-induced dysregulation of histones and histone-regulating proteins. In an effort to understand the carcinogenic potential of BDE-209, the first such study found that HEK293T cells exposed to micromolar range levels of the toxin exhibited altered expression of chromatin-regulating genes, specifically a histone gene cluster that the authors hypothesize could affect nucleosome properties (Li *et al.* 2014). In the same year, another group reported that exposure of the marine madaka (*Oryzias melastigma*) to BDE-47 led to sex-specific differential protein expression in male and female gonads, with several histone variants (H2b, H3.3, H3a, H2a) being down-regulated in male gonads (Fong *et al.* 2014). Another study found that exposing maize (*Zea mays* L.) to BDE-47, and its metabolites 6-OH-BDE-47 and 6-MeOH-BDE-47, led to elevated levels of ROS and phospho-H2AX, likely in response to DNA damage. Interestingly, the hydroxylated metabolite produced the most severe effects (Xu *et al.* 2015). Another study, primarily concerned with the relationship of PBDE exposure to reproductive health, exposed pregnant rats to BDE-47 from E8 to PND21. Male offspring were then assessed at PND120 for alterations in testes. It was found that exposed rats had smaller testes, decreased sperm production, and interestingly, an altered testes transcriptome and 4-fold decrease in protamine and transition gene expression (proteins responsible for histone-protamine exchange) (Khalil *et al.* 2017). Aside from these data that indicate potential disruptions of histone expression and nucleosome alteration, there are two studies that report PBDE-induced dysregulation of chromatin-regulating proteins. The first found that BDE-47 treatment downregulated SirT1 expression (a histone deacetylase) in the livers of mice due to NAD(+)-depletion (Zhang *et al.* 2015).

The potential importance of understanding the effects of PBDEs on chromatin dynamics cannot be understated given the fundamental importance of chromatin properties for regulating gene expression and thus cellular states. Going forward, it will be important for investigators to focus on identifying additional effects on chromatin while distinguishing those that are direct from indirect, hopefully allowing for elucidation of the underlying mechanism.

1.2.4 Other Affected Epigenetic Mechanisms (Non-Coding RNAs)

Non-coding RNAs—such as long non-coding RNA (lncRNA) and microRNA (miRNA)—can also act as epigenetic regulators (Rinn and Chang 2012; Cech and Steitz 2014). Various PBDE exposures have been reported to alter expression of miRNAs, and one study described effects on expression of liver lncRNAs. The earliest study assessing miRNA expression as an endpoint following PBDE exposure utilized placental samples collected from the National Children's Study. Among other associations established for PCB and heavy metal exposures, the study reported a positive association between BDE-209 and miR-188-5p expression and an inverse association for BDE-99 and let-7c (Li et al. 2015). Another group exposed hESCs in vitro to low doses of BDE-209 (1,

10, 100nM) for 4 days, inducing apoptosis and downregulating pluripotency genes, particularly OCT4, in part by hypermethylation of the promoter and induction of miR-145/335 which repress OCT4. There was also generation of ROS and decreased superoxide dismutase (SOD2) expression. ROS and OCT4 effects were partially rescued by treatment with the antioxidant NAC (Du et al. 2016). An even more recent study employing human cells found that, after stimulating THP-1 macrophages with BDE-209 and LDL, there was dose-dependent repression of miRNA-21 which subsequently de-repressed toll-like receptor 4 expression (TLR4), enhancing TLR4-dependent lipid uptake (Zhi et al. 2018; Zhi et al. 2019b; Zhi et al. 2019a).

In addition to these examples in humans, two rodent studies concerning non-coding RNAs in the liver have been published. The first found that BDE-47 exposure upregulates CYP3A1 in rat liver and that this upregulation is mediated by BDE-47-induced repression of miRNA-23b, which negatively regulates CYP3A1 mRNA via a 3' UTR binding site (Sun *et al.* 2016). The other study reported that conventional and gut-microbiome-depleted mice exhibit dysregulated lncRNA expression in liver tissue in response to both BDE-47 and BDE-99 exposure (Li and Cui 2018). Interestingly, BDE-47 has also been found to induce dysregulation of novel miRNAs in exposed zebrafish larvae. Of particular interest is miR-735, which may play essential roles in larval sensory development, explaining previously observed BDE-47-induced disruption of zebrafish visual perception (Zhao *et al.* 2017a). In the near future, a general model of PBDE-induced miRNA dysregulation may hopefully be established given the multiple intriguing examples already characterized.

1.3 Summary

Considering this growing body of work documenting epigenetic dysregulation induced by PBDE exposure, there appear to be several central lines of evidence emerging from research done in various health contexts, including: adipocyte differentiation and obesity, reproductive health—of both sperm/testes and the placenta, carcinogenicity (especially thyroid related), and negative impacts on nervous system formation and function. It is becoming clear that many, if not all, of these various aspects of human health are impacted by PBDE-induced disruption of normal epigenetic states and mechanisms.

There are fairly consistent findings of a negative relationship between PBDE levels and DNA methylation from *in vitro* and non-human animal studies across varied cell/tissue types and methylation detection methods. However, the data from human samples is more difficult to interpret. Studies reporting effects on global DNA methylation levels inferred from representative regions have incongruent results, and evidence of alterations to methylation in the placenta are, likewise, not in direct agreement. However, this confoundment and the fact that human studies have so far been conducted across very different populations

and models should only encourage further work on the topic, especially given indications from non-human animal and *in vitro* studies. It will be of great value if these types of studies can build on the tentatively established negative impact of PBDEs on methylation and begin to focus on understanding the mechanisms underlying the alterations, while continuing to clarify effects in human studies.

Compared to DNA methylation, the literature is sparser regarding the effects of PBDEs on other epigenetic mechanisms such as chromatin dynamics and expression of non-coding RNAs. However, some interesting ideas are beginning to emerge. While not yet well understood, PBDE-induced dysregulation of histones and chromatin regulators is an intriguing intersection for PBDEs and neurodevelopmental disorders, bolstered by the recent emergence of chromatin regulation as a major node of NDD risk (Lasalle *et al.* 2013; Gabriele *et al.* 2018). Further, it is tempting to speculate that epigenetic effects of PBDE exposure may, generally, turn out to be a point of convergence for environmental and genetic factors that contribute to NDDs. If the effects of these compounds on targets such as DNA methylation, chromatin components and regulators, and non-coding RNA expression (all of which are mechanisms known to have roles in neurodevelopment and perhaps NDD etiology) can be further explored and resolved, one or more could very well turn out to be that link. This is of pressing importance, especially for neurodevelopmental disorders considering their explosive increase in prevalence, growing evidence for the involvement of PBDEs in their etiology, and the long elusive role of environmental factors in these devastating conditions.

A major challenge for epigenetic PBDE research will be to assimilate new findings into the existing framework of PBDE toxicity that has been established from insights into other major impacted biological mechanisms. It will also be important to carefully consider nuanced aspects of exposures including tissue and sub-cellular localization, conduct more research on environmentally relevant doses and mixtures of PBDEs, further explore the prevalence and effects of their metabolites, and, to the extent that it is possible, integrate evidence generated across human and non-human studies (both *in vitro* and *in vivo*). This will be necessary in order to construct a more holistic understanding of how these compounds impact cellular states and, ultimately, phenotypic outcomes. By moving towards such an understanding with continued research, we may eventually be able to explain how and to what extent these pervasive environmental pollutants are related to the numerous human health conditions that they appear to be contributing to and may perhaps gain additional insight into the nature of PBDE-related health complications themselves.

With the emerging bodies of evidence for PBDE-induced epigenetic disruptions, epigenetic regulation (particularly chromatin remodeling) as a major node of risk in NDDs, and the growing evidence linking PBDE exposure levels with NDD-related behavioral abnormalities in humans and other animal models,

in my dissertation research I sought to characterize the cellular effects of PBDE exposures and related underlying molecular mechanisms in the developing brain. Specifically, I first tested the hypothesis that the effects of nanomolar range exposures to BDE-47 and its hydroxylated metabolites interact with epigenetic regulation in neurons from the developing nervous system (Chapter 2), subsequently developed and tested a mechanistic hypothesis of synaptic disruption and intracellular signaling inhibition by ortho-hydroxylated PBDE metabolites (Chapter 3) based directly on findings generated from the data presented in Chapter 2– leading to an appreciation that hydroxylated PBDE metabolites likely affect multiple levels of cellular function by interfering with molecular processes in a sub-cellular-specific manner, and finally to the generation of several hypotheses integrating these new findings with each other and with data from the diverse biological fields surrounding PBDE neurotoxicity (Chapter 4).

Chapter 2: Persistent 6-OH-BDE-47 exposure impairs functional neuronal maturation and alters expression of neurodevelopmentally-relevant chromatin remodelers

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Environmental Epigenetics 2018

2.1 Abstract

Polybrominated diphenyl ethers (PBDEs) are a pervasive class of brominated flame retardants that are present in the environment at particularly high levels, especially in the United States. Their environmental stability, propensity for bioaccumulation, and known potential for neurotoxicity has evoked interest regarding their effects on the developing nervous system. Exposure to PBDEs has been strongly associated with neurodevelopmental disorders. However, the details of their mechanistic roles in such disorders are incompletely understood. Here, we report the effects of one of the most prevalent congeners, BDE-47, and its hydroxylated metabolites on the maturation and function of embryonic rat cortical neurons. Prolonged exposure to 6-OH-BDE-47 produces the strongest effects amongst the parent BDE-47 congener and its tested hydroxylated metabolites. These effects include: i) disruption of transcriptional responses to neuronal activity, ii) dysregulation of multiple genes associated with neurodevelopmental disorders, and intriguingly, iii) altered expression of several subunits of the developmentally-relevant BAF (Brg1-associated factors) chromatin remodeling complex, including the key subunit BAF170. Taken together, our data indicate that persistent exposure to 6-OH-BDE-47 may interfere with neurodevelopmental chromatin remodeling mechanisms and gene transcription programs, which in turn are likely to interfere with downstream processes such as synapse development and overall functional maturity of neurons. Results presented in this chapter have identified a novel aspect of 6-OH-BDE-47 toxicity and open new avenues to explore the effects of a ubiquitous environmental toxin on epigenetic regulation of neuronal maturation and function.

Keywords: neurodevelopment, BDE-47 exposure, Arc, activity-induced transcription, BAF complex

2.2 Introduction

An increasingly studied point of convergence that may help to provide new insight on the relationship between the various effects of PBDE exposures and adverse behavioral outcomes is epigenetic regulation, specifically of gene transcription during neurodevelopment. Epigenetic mechanisms are a major driving force of normal neurodevelopment (Lasalle *et al.* 2013), and many neurodevelopmental complications involve dysregulation of gene transcription (Parikshak *et al.* 2015; Vissers *et al.* 2015). Although several epigenetic components of neurodevelopment are now well known, there remains much more to be characterized (Sweatt 2013), especially in regards to how they interact with environmental challenges like PBDE exposure.

Epigenetic regulation refers to a range of processes (detailed more in the Chapter 1) including chemical modifications to DNA and histone proteins, non-coding RNA expression, and ATP-dependent remodeling of chromatin structure. The latter is of particular interest in the context of NDDs, as it has recently emerged as a major node of risk including several highly penetrant mutations associated with the disorders (Gabriele *et al.* 2018). ATP-dependent chromatin remodeling is a process by which regulatory proteins restructure chromatin–the complex of DNA and proteins making up chromosomes in eukaryotic cells–a process that plays a major role in facilitating the vast diversity of cellular types and function produced from an organism's genome (Ho and Crabtree 2010). The general mechanisms of how these regulators interact with DNA and histones/nucleosomes to modify their composition and structure is thought to be largely similar. However, the various canonical chromatin remodeling protein complexes are composed of many subunits and are regulated by various interacting proteins, including cell-type specific factors (Clapier *et al.* 2017).

One such dynamically-regulated remodeling complex that is known to play critical roles in neurodevelopment is the BAF (Brg1-associated factors) complex (Staahl and Crabtree 2013; Sokpor *et al.* 2018). BAF complexes containing neuron-specific subunits are known to instruct neurodevelopment, especially the differentiation of neurons (Staahl and Crabtree 2013), as well as the regulation of synaptic formation, plasticity, and learning and memory (Vogel-Ciernia *et al.* 2013; Zhang *et al.* 2016). Interestingly, mutations in chromatin remodelers, including BAF complex subunits, have recently been identified as NDD risk factors, establishing a major node of risk in the complex network of genetic factors now associated with NDDs (further details and references in Chapter 1).

Considering this, we sought to explore the ways in which exposure to BDE-47 and its hydroxylated metabolites influence the maturation and function of neurons from the developing nervous system, and screened for possible intersections with NDD risk at the level of transcriptional dysregulation by measuring expression of NDD candidate genes representing several of the major nodes of NDD risk (Vissers *et al.* 2015; Iakoucheva *et al.* 2019)– intracellular signaling molecules, synaptic proteins, and epigenetic regulators including several BAF complex subunits.

2.3 Materials and Methods

2.3.1 Plasmids and sub-cloning

A commercial shRNA construct for BAF170 (CCCAAACTGCTAGGGAAATTA) was obtained from Sigma. This shRNA sequence was inserted into pLKO.1-puro (designed by RNAi consortium or TRC; obtained from Addgene) and then packaged into lentiviruses. Self-inactivating HIV lentivirus particles were produced by transfecting 293T cells with the shRNA vector, envelope (pMD2.G; Addgene), and packaging plasmids (psPAX2; Addgene) using a previously described protocol(Saha *et al.* 2011). The BAF170 expression construct in a lentiviral backbone was a kind gift from Dr. Trevor Archer (NIEHS, NIH)(Wade *et al.* 2016). BAF170 expression from this construct was validated by Western blotting.

2.3.2 Dissociated neuronal culture, RNAi and cell treatment

Cultures of cortical neurons were prepared from embryonic day 18 Sprague Dawley rats (UC Merced IACUC approval: AUP#13-0007 and AUP#16-0004). Dissociated cortical neurons were plated in Neurobasal medium (Invitrogen) supplemented with 25 μM glutamate (Sigma-Aldrich) and 0.5 mM L-glutamine (Sigma-Aldrich) and either B27 (Invitrogen) or NS21 and maintained in a similar medium without glutamate. NS21 was prepared in the laboratory as previously described (Chen *et al.* 2008). Neurons were routinely used for induction assays between 10–16 days *in vitro*. For infection with recombinant lentiviruses, the viral supernatant was diluted in neuronal media and cells were infected at a multiplicity of infection ranging from 2 to 5. To induce gene transcription under basal conditions using synaptic circuits, we co-treated neurons with 50μM Bicuculline (Sigma-Aldrich) and 75μM 4-Aminopyridine (Acros Organics) (Papadia *et al.* 2005). To induce gene transcription extra-synaptically, we blocked activity with 1μM TTX (Calbiochem) and induced the MAP-kinase pathways with 1μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) (Schultz *et al.* 1997). BDE-47 and metabolites used to treat cultures were obtained from AccuStandard (BDE-047N, HBDE-4003N, HBDE-4004N, HBDE-4005N).

2.3.3 RNA extraction and gene transcription quantitation

Total RNA was isolated from dissociated neurons using the GeneJET RNA Purification Kit (Thermo) with an off-column DNase (Promega) digestion. cDNA was synthesized using MuLV reverse transcriptase (Promega), random primers (Promega), oligo dTs (Promega), and RNase inhibitors (Thermo Scientific). Quantitative real-time PCR (qRT-PCR) was performed to quantify mRNA levels of specific transcripts using iTaq Universal Sybr Green Supermix (BioRad) and the BIO-RAD CFX Connect realtime PCR Detection System. Pre-mRNA levels

were estimated as previously described (Saha *et al.* 2011).

2.3.4 Sample preparation for electrophoresis

Neurons were lysed in ice-cold 1X RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Na- deoxycholate, 0.1% SDS, 0.1% NP-40) supplemented with 1:100 protease inhibitor cocktail (Sigma-Aldrich-Aldrich). Lysed neurons were sheared by sonication (low setting; three cycles on Bioruptor®), cell debris pelleted at 15,000 rpm for 5 minutes at 4°C, and clarified supernatant transferred to pre-chilled 1.5 mL microcentrifuge tube. Total cell extracts were denatured at 95°C, for 5 minutes, using either home-made 5X Laemmli buffer, 2X-, or 4X-Laemmli sample buffer (both from BIO-RAD).

2.3.5 Western blotting and imaging

Denatured protein samples were resolved on 4-20%- (BIO-RAD cat. no. 4568095) or 4-15%- (BIO-RAD cat. no. 456-1083) Mini PROTEAN® gels in Tris/Glycine/SDS (BIO-RAD cat. no. 1610772). Resolved proteins were transferred onto LF PVDF membrane, using the BIO-RAD TBT RTA kit and protocol (cat. no. 1704272). PVDF membranes were incubated at 4°C overnight with appropriate primary antibodies in 1X TBS-T with 0.5% BSA at 1:1000 dilution. Primary antibodies included the following antibodies: β-Actin (ThermoFisher Scientific AM4302), BAF170 (CST 12760), BAF155 (CST 11956), Brg1 (CST 49360), BAF47 (CST 91735). Next day, membranes were washed three times in 1X-TBST, probed with appropriate Alexa Fluor® secondary antibodies (Life Technologies) for 40 minutes at room temperature, washed three times with 1X TBS-T, and imaged using BIO-RAD Multiplex ChemiDoc™ Imaging System.

2.3.6 Immunocytochemistry and microscopy

Antibodies for immunocytochemistry were used at dilutions between 1:100-1:500 and include the following: NeuN (Millipore ABN78), Doublecortin (CST 4604S), Nestin (Invitrogen MA1-91657). Infected neurons were washed twice with 1X ice-cold PBS (Fisher Sci). The cells were then incubated with 4% paraformaldehyde (Sigma-Aldrich) in 1X PBS for 15 minutes at room temperature and then washed twice with 1X PBS, permeabilized at room temperature for 20 minutes with 0.5% Triton X-100 (Fisher Sci), washed twice and blocked for 30 minutes with 10% goat serum (Gibco) in 1X PBS. Cells were incubated at 4°C overnight in 3% goat serum in 1X PBS with primary antibodies. Next day, primary antibody solution was removed and cells were washed thrice with 0.05% Tween (Fisher) in 1X PBS (0.05% PBS-T), and incubated with appropriate Alexa Fluor® secondary antibody (Lifetech) for 45 minutes, washed thrice with 0.05% PBS-T, cured overnight with Prolong Anti-Fade Gold with DAPI and imaged. Images were

captured with a Keyence BZ9000-E microscope at 40X magnification.

2.3.7 Cell viability assay

Cell viability was assessed by an MTT assay (Biotium) (Mosmann 1983) wherein mitochondrial activity is detected colorimetrically following incubation of cells with a tetrazolium salt. The assay was conducted according to manufacturer's instruction, except that MTT incubation time was shortened to 30 min to avoid reaching a plateau where differences in product formation would be indistinguishable and reagent volumes were proportionally scaled up to appropriate amounts for 24-well plates.

2.3.8 Statistics

Error bars represent standard error of mean throughout this article. Statistical comparison of datasets was performed by one way ANOVA with Fisher's LSD (Figure 2C and 6A) or by two way ANOVA with Tukey's post hoc test (all other figures). Biological replicates are indicated throughout as *N* in corresponding figure legends. Biological replicates constitute cell culture preparations from independent dams.

2.4 Results

2.4.1 Characterization of E18 primary rat neuronal cultures

We dissected brain tissue from the pups of timed-pregnant Sprague-Dawley rats (*Rattus norvegicus*) on embryonic day 18 (E18) to obtain dissociated cortical neurons. Cells were then plated as monolayers in supplemented Neurobasal growth medium. The identity of these cultured cells was characterized over the first week of growth by immunocytochemistry (ICC) using antibodies raised against Nestin, DCX, and NeuN (neural progenitor, differentiating neuron, and differentiated neuronal markers, respectively (Frederiksen and McKay 1988; Gleeson *et al.* 1999; Mullen *et al.* 1992) (**Fig. 1**). The cultures were composed of

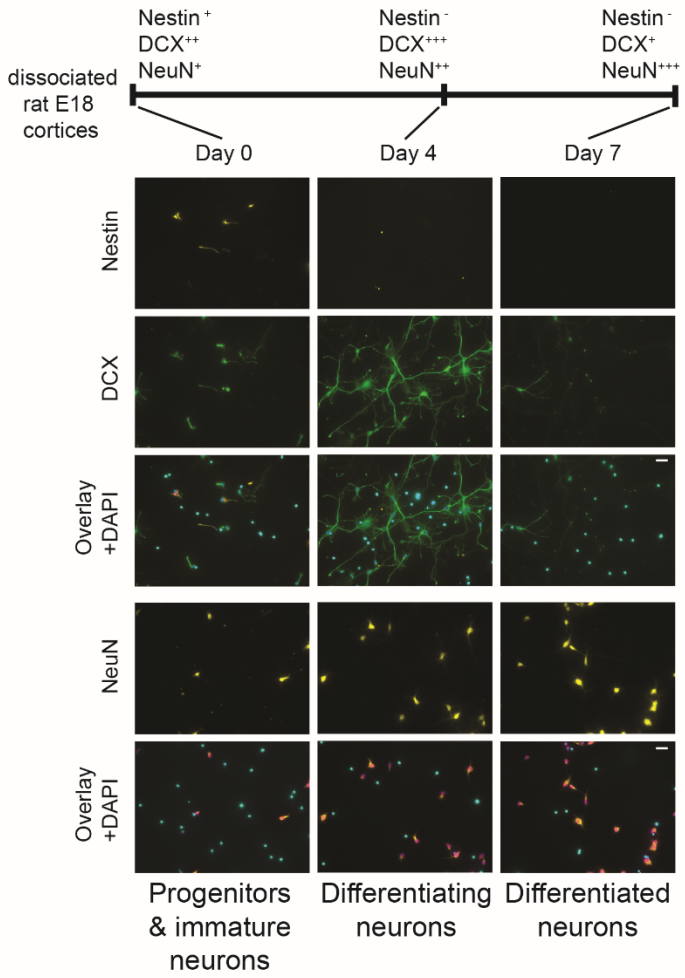


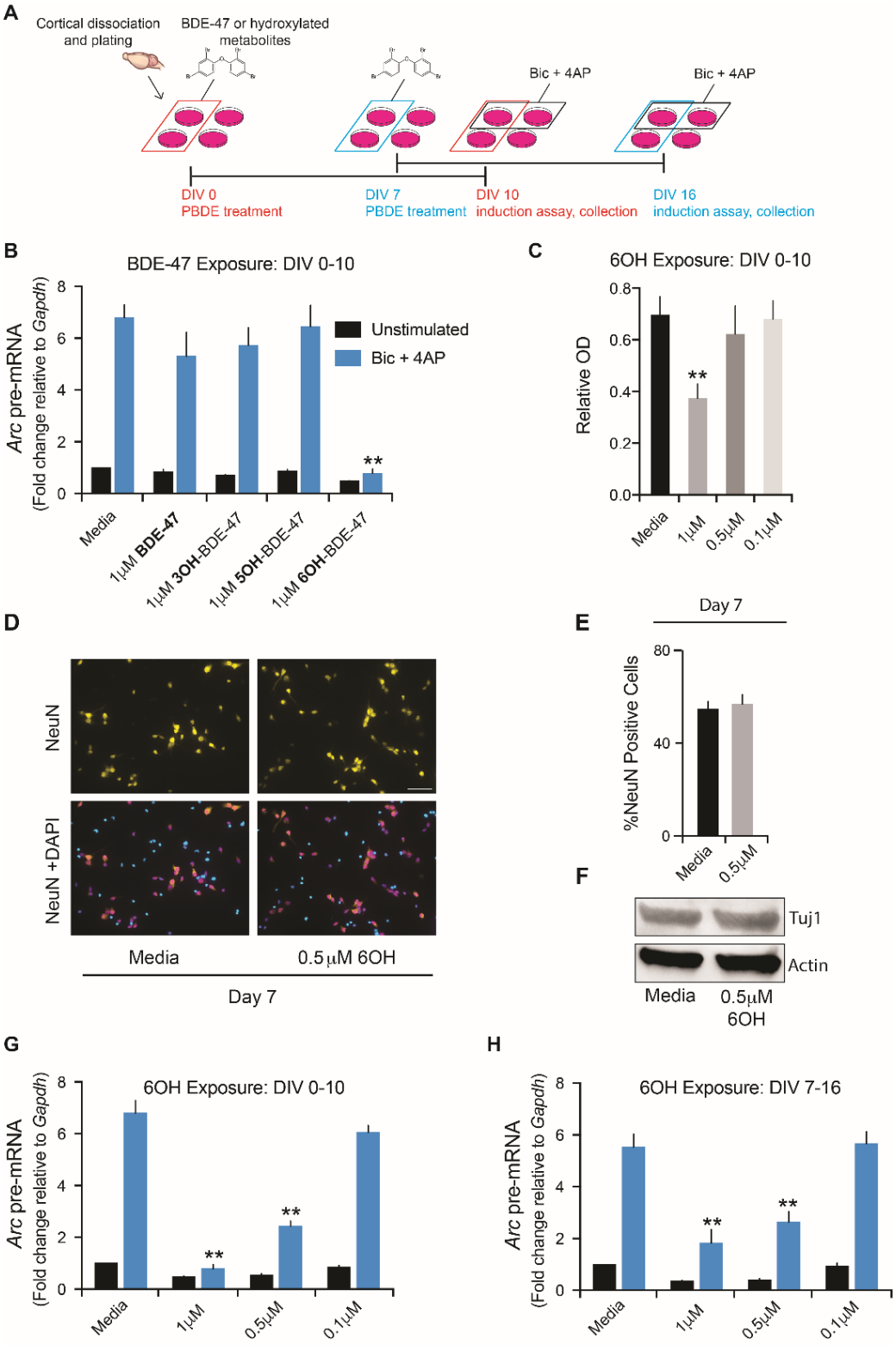
Figure 2-1. Characterization of neuronal cultures across the first week of growth by immunocytochemistry. Rat cortical neurons were obtained by dissecting and dissociating cortices from E18 pup brains. Cultures derived from these cells were stained using antibodies against several markers of neuronal maturation: Nestin (neuronal precursor), DCX (differentiating neurons), and NeuN (differentiated neurons). Staining was conducted at indicated time points. Scale bar = 25μm. N=3

cells exhibiting expression of: Nestin and DCX on day 0 *in vitro* (DIV 0), predominantly DCX expression by DIV 4, and largely NeuN expression by DIV 7. Based on the expression of these markers, we identified our DIV 0 cells to be a mixture of both neuronal progenitors and differentiating neurons, whereas DIV 4 and DIV 7 cells were predominantly differentiating and differentiated neurons respectively. Utilizing cultures of this nature allowed us to investigate the effects of exposure to BDE-47 and its hydroxylated metabolites across various early stages of neuronal maturation *in vitro*.

2.4.2 6-OH-BDE-47 impairs neuronal maturation and activity-induced gene transcription

Mature neurons sense environmental cues through neuronal activity and can respond via activity-induced gene transcription (Flavell and Greenberg 2008). Therefore, we sought to test the effects of PBDE exposure on functional maturation by conducting neuronal activity-induced gene transcription assays using a widely studied, neuron-specific immediate early gene (IEG), *Arc*, as a readout (referred to from here on as 'activity-induced gene transcription assay'). Assays were conducted in cultures that were chronically exposed to 1μM of BDE-47 or one of its hydroxylated metabolites– 3OH-BDE-47 (3OH), 5OH-BDE-47 (5OH), and 6-OH-BDE-47 (6-OH) and neuronal activity was induced using Bicuculline and 4AP (Bic+4AP). *Arc* pre-mRNA, the direct output of transcription, was detected at fifteen minutes after induction and quantified by quantitative real-time PCR (qRT-PCR) using intron-exon spanning primers. We found that transcription of *Arc* was significantly attenuated in cultures exposed to 6-OH from DIV 0-10, but not in cultures exposed to other compounds (**Fig. 2B**) suggesting that chronic exposure to 6-OH may interfere with functional maturation of neurons.

Figure 2-2. **(next page)** Chronic 6OH exposure is detrimental to neuronal maturation and function. Activity induced Arc expression assays were conducted by treating cultures with Bic and 4AP for 15 min. (A) Timeline depicting PBDE exposure durations leading up to activity induction assays. Ten-day exposures were started either concurrently with plating (DIV 0) or after a week of growth (DIV 7) to assess differences in effect on differentiating versus differentiated neurons. (B) Induction of Arc after a 10-day exposure to 1μM BDE-47 or one of its hydroxylated metabolites, N=3. (C) Assessment of cell viability following 10 days of 1μM 6OH-BDE-47 exposure. Viability was assessed colorimetrically by MTT assay. N=3. (D) Representative images of NeuN and DAPI stained cells on DIV 7 following treatment with 0.5μM 6OH starting at DIV 0. Scale bar = 50μm (E) Quantification of NeuN and DAPI staining represented in D, (2, 562 untreated and 2, 634 6OH treated cells counted). (F) Representative western blot for Tuj1 (a marker of differentiated neurons) in cells exposed to 0.5μM 6-OH-BDE-47 for seven days, N=3. (G) Induction of Arc after a ten-day exposure to various concentrations of 6OH, N=3. (H) Similar assay as shown in (G) except 6OH exposure was started on DIV 7, N=4. * $P < 0.05$, ** $P < 0.01$. ns = non-significant


A
Cortical dissociation and plating
BDE-47 or hydroxylated metabolites
Bic + 4AP
Bic + 4AP
DIV 0
PBDE treatment
DIV 7
PBDE treatment
DIV 10
induction assay, collection
DIV 16
induction assay, collection
B
BDE-47 Exposure: DIV 0-10
Arc pre-mRNA
(Fold change relative to Gapdh)
Unstimulated
Bic + 4AP
**
Media
1μM BDE-47
1μM 3OH-BDE-47
1μM 5OH-BDE-47
1μM 6OH-BDE-47
C
6OH Exposure: DIV 0-10
Relative OD
**
Media
1μM
0.5μM
0.1μM
D
NeuN
NeuN +DAPI
Media
0.5 μM 6OH
Day 7
E
Day 7
%NeuN Positive Cells
Media
0.5μM
F
Tuj1
Actin
Media
0.5μM 6OH
G
6OH Exposure: DIV 0-10
Arc pre-mRNA
(Fold change relative to Gapdh)
**
**
Media
1μM
0.5μM
0.1μM
H
6OH Exposure: DIV 7-16
Arc pre-mRNA
(Fold change relative to Gapdh)
**
**
Media
1μM
0.5μM
0.1μM

Lack of functional maturation could be an inadvertent fallout of 6-OH-induced neurotoxicity or impaired neuronal differentiation. Any cytotoxicity in cells exposed to different doses of 6-OH was measured by MTT assay. This range of doses was selected based on an approximation of environmentally relevant exposures. The reasoning and justification for this approximation is detailed further in the discussion. While 1μM 6-OH was found to induce a significant reduction in cell viability after chronic exposure for ten days, 0.5 and 0.1μM 6-OH doses did not (**Fig. 2C**). Next, we studied effect of 6-OH on neuronal differentiation. 6-OH has been previously shown to inhibit differentiation at lower concentrations than its parent compound in adult neural progenitor cells and human neuronal precursors (Schreiber *et al.* 2010; Li *et al.* 2013). To verify any effect of 6-OH on neuronal differentiation in our system, we studied neuronal fate markers (NeuN and Tuj1) in DIV 7 neurons after prolonged exposure to the non-cytotoxic 0.5μM 6-OH dose (**Fig. 2D-F)**. On DIV 7, when neurons are differentiated in our system (Fig. 1), there was no difference in fraction of NeuN positive neurons between untreated and 6-OH treated cultures (**Fig. 2D, E**). Similar results were obtained with Tuj1, another marker for differentiated neurons (**Fig. 2F**), indicating that neurons can attain their fate and differentiate irrespective of exposure to 6-OH in our culture conditions. Therefore, reasons for 6-OH exposure-related lack of functional maturation (**Fig. 2B**) likely lie elsewhere.

Next, to explore any differences in 6-OH effects between differentiating and differentiated neurons, we conducted additional activity-induced gene transcription assays, exposing cells to a range of 6-OH concentrations from both DIV 0 and DIV 7. From these assays, a dose-dependent activity-induced *Arc* transcriptional response was observed in cultures exposed at both DIV 0 and DIV 7 (**Fig. 2G, 2H**). Interestingly 0.5μM 6-OH exposure, which does not significantly affect cell viability, produced effects that were not statistically different from 1μM exposure. Taken together, these results indicate that chronic 6-OH exposure impairs functional neuronal maturation while differentially affecting cell viability depending on the exposure dose.

2.4.3 6-OH-BDE-47 exposure impairs synaptic and extra-synaptic modules of activity-induced transcription

Transcription of IEGs in response to neuronal activity relies on a cascade of intracellular components starting with functional synapses, then transduction of signals from the synapse to the nucleus, and finally chromatin accessibility in the nucleus itself (Matamales 2012; Saha and Dudek 2013). As Bic+4AP treatment induces neuronal activity by GABAergic disinhibition, any of these factors affected by 6-OH could be contributing to the decreased production of nascent *Arc* pre-mRNA in Figure 2. As such, results from Bic+4AP induction assays potentially reflect an accumulation of effects arising from dysregulation at any point from the synapse to the nucleus. Therefore, to more specifically test if 6-OH

was acting at synapses or further downstream, we induced *Arc* transcription intracellularly at the signal transduction level by stimulating the Mitogen Activated Protein Kinase (MAPK) pathway, which is known to transduce signals for rapid neuronal gene transcription (David Sweatt 2001; Chotiner *et al.* 2010). Our laboratory has established that neuronal rapid IEGs, including *Arc* (Saha *et al.* 2011), may be induced extrasynaptically by activating the MAPK pathway with 1μM of the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) while blocking neuronal activity with 1μM tetrodotoxin (TTX), a potent sodium channel blocker (**Fig. 3A**) (Dunn *et al.* 2017). Using this extra-synaptic induction protocol, we found that *Arc* is induced within fifteen minutes. This *Arc* pre-mRNA induction was inhibited significantly by 1μM, but not 0.5μM or 0.1μM,

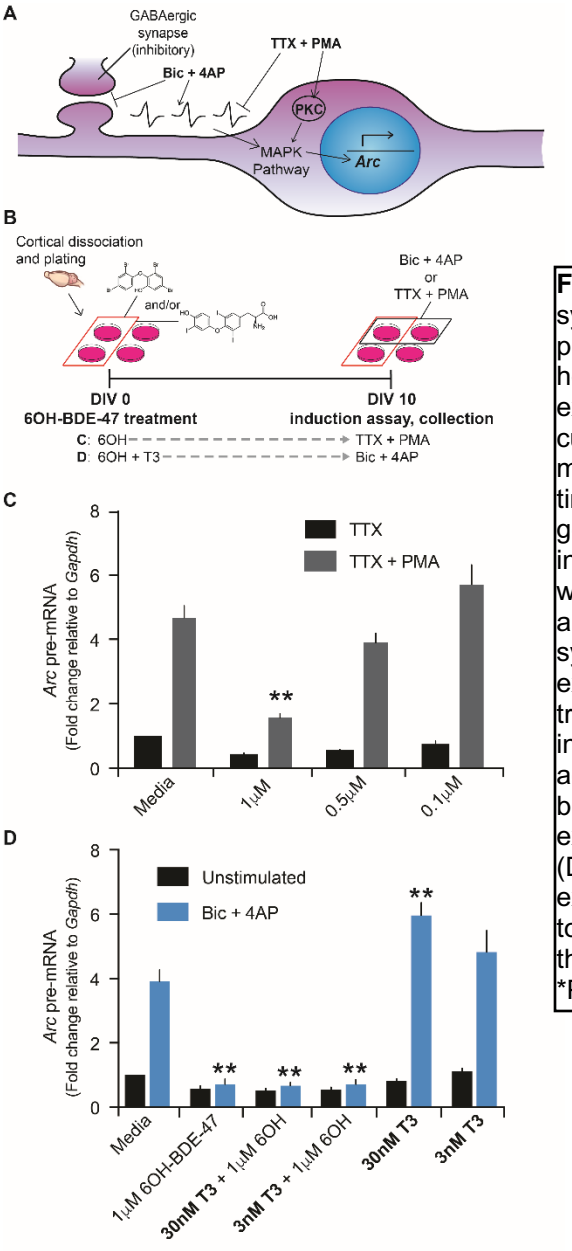


Figure 2-3: Effects of 6OH exposure are at the synaptic and extrasynaptic levels and are not primarily mediated by disruption of thyroid hormone signaling. Activity induced Arc expression assays were conducted by treating cultures with Bic and 4AP or TTX and PMA for 15 min after ten days of 6OH exposure starting at the time of plating. (A) Depiction of different modes of gene transcription assays. Note: Bicþ4AP induction (Fig. 2) is reliant on synaptic activity whereas TTXþPMA induction bypasses synapses and jumpstarts signaling cascades extra-synaptically. (B) Timeline depicting 6OH exposures with or without addition of triiodothyronine (T3) followed by various activity induction assays. (C) Induction of Arc via direct activation of the MAPK pathway with PMA while blocking membrane activity with TTX in cultures exposed to various concentrations of 6OH N=5. (D) Induction of Arc with Bicþ4AP following exposure to 6OH with and without additions of T3 to outcompete potential interactions of 6OH with thyroid hormone transporters and receptors N=3. *P < 0.05, **P < 0.01

6-OH exposure (**Fig. 3C**). Although 1μM 6-OH exposure caused a certain amount of cell death (**Fig. 2C**), the effect of the same dose seen in this assay may not be entirely due to neurotoxicity because the *Arc* induction values are normalized by an internal control (*Gapdh*, representing live cells). Additionally, comparing Fig. 2G and 3C, our data indicate that prolonged exposure to non-lethal dose of 6-OH (0.5μM) may impair synaptic formation and/or functions (Bic+4AP assays; Fig. 2G), but spares extra-synaptic cascades of events that lead up to *Arc* transcription (TTX+PMA assays; Fig. **3C**).

One major suspected mode of action for PBDE toxicity is dysregulation of thyroid hormone homeostasis, including disruption of thyroid hormone levels, transport, and receptor activity (Costa *et al.* 2014). These effects are thought to be mediated by structural similarity between PBDEs and thyroid signaling molecules (Dingemans *et al.* 2011). To test if the observed effects of 6-OH in our assays are mediated primarily by disruption of intracellular thyroid signaling, cultures were co-exposed to 1μM 6-OH and either 30 or 3nM triiodothyronine (T_3), which is present at low levels in culture media already. Elevated levels of T_3 were shown to rescue 10μM BDE-47-induced reduction of neuronal migration in another study(Schreiber *et al.* 2010). In our assays, elevated levels of T_3 did not rescue 1μM 6-OH-induced attenuation of *Arc* induction following Bic+4AP stimulation, although the addition of 30nM T_3 alone did significantly enhance induced *Arc* transcription (**Fig. 3D**). Together, these results indicate that elevated levels of thyroid hormone cannot rescue effects of 6-OH exposure, perhaps including its neurotoxic effects, opening the possibility of a thyroid-hormone pathway-independent mechanism of 6-OH toxicity.

2.4.4 BDE-47 and its hydroxylated metabolites dysregulate expression of NDD candidate genes

Observing that chronic exposure to 6-OH disrupts neuronal activity-related function in dosage-dependent manners, and that the effects are not primarily mediated by disruption of thyroid hormone homeostasis, we sought to identify other potential mechanisms. To accomplish this, we screened 15 NDD candidate genes, coding for proteins of various functions important for neuronal development and function (**Table 1**), for changes in mRNA levels following exposure to 1μM BDE-47 or its hydroxylated metabolites. Exposures were started at various time points to assess effects during neuronal differentiation. We found that exposure to BDE-47, 3OH, and 5OH altered mRNA levels of only a few genes, while 6-OH exposure altered mRNA levels of 11 out of 15 genes at the earliest and middle time points (exposures at DIV 0 and DIV 4), and a smaller subset when differentiated neurons were exposed starting at DIV 7 (**Fig. 4**). This observation suggests that 6-OH exposure may have temporally specific effects, with immature neurons being affected to a seemingly greater extent. Following up on the widespread effect caused by 6-OH, we measured mRNA levels of the

Gene	Description/Function	Associated Brain Disorders
Adnp	Activity-Dependent Neuroprotective Protein: zinc finger transcription factor that modulates p53 activity and interacts with BAF chromatin remodeling complexes to regulate transcription	ASD, ID
Arid1b	AT-Rich Interaction Domain 1B (BAF250b): DNA interacting protein, subunit of the neural precursor and neuron specific BAF chromatin remodeling complexes	ASD, ID
Cbx4	Chromobox 4: component of a Polycomb group (PcG) PRC1-like complex which is involved in transcriptional repression of many genes throughout development and mediates monoubiquination of histone 2A lysine 119 residues	ASD
Ctnnb1	Catenin Beta 1: component of the canonical Wnt signaling pathway, coactivator of TCF/LEF transcription factors that activate Wnt responsive genes which play diverse neurodevelopmental roles such as central nervous system patterning, and neural stem and precursor cell proliferation	ASD, ID
Dyrk1a	Dual Specificity Tyrosine Phosphorylation-Regulated Kinase 1A: a nuclear protein kinase that catalyzes autophosphorylation of serine/threonine and tyrosine residues, thought to play a role in regulating signaling pathways contributing to cell proliferation and neurodevelopment	ASD, ID
Med13l	Mediator Complex Subunit 13 Like: a subunit of the mediator complex, a transcriptional coactivator for RNA polymerase II transcribed genes that is recruited to promoters by binding with regulatory proteins thus serving as a scaffold for the preinitiation complex, involved in early neurodevelopment	ASD, ID
Ncor1	Nuclear Receptor Corepressor 1: corepressor of thyroid-hormone and retinoic-acid receptor target genes, known to recruit histone deacetylases that promote the formation of condensed chromatin structure that prevents access of transcription factors and thus represses transcription	ASD, HD
Npas2	Neuronal PAS Domain Protein 2: transcription factor that is a core component of the circadian clock, also known to regulate the transcription of metabolism, cell cycle, and DNA repair related genes	ASD
Phf2	PHD Finger Protein 2: a zinc-finger-like plant homeodomain containing demethylase that acts on both histone and non-histone proteins, dimerizes with Arid5b at target promoters and demethylates histone 3 lysine 9 (H3K9me2) residues leading to transcriptional activation	ASD
Rps6ka2	Ribosomal Protein S6 Kinase A2: a member of the RSK family of serine/threonine kinases that phosphorylates members of the mitogen-activated protein kinase signalling pathway, regulates processes such as cell growth, survival, and proliferation	ASD
Shank3	SH3 and Multiple Ankyrin Repeat Domains 3: major scaffold protein of the postsynaptic density that organizes neurotransmitter receptors, ion channels, and other membrane proteins via interactions with the actin cytoskeleton, plays a role in dendritic spine maturation as well as synaptic formation and plasticity	ASD, ID, SCZ
Smarcc2	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily C Member 2 (BAF170): subunit of the neural precursor and neuron specific BAF chromatin remodeling complexes, thought to be a scaffolding subunit required for stabilization of other BAF subunits, involved in transcriptional activation and repression, known to regulate cortical thickness and neural progenitor proliferation	ASD, SCZ
Sp1	Specificity Protein 1: a zinc finger transcription factor that binds GC-rich motifs at many promoters, involved in many cellular processes such as differentiation, growth, apoptosis, and recruitment of chromatin remodeling enzymes including Brg1 and Brg associated factors to regulate transcription	ASD
Tbl1xr1	Transducin Beta Like 1 X-Linked Receptor 1: thought to be a component of the NCoR and HDAC3 repressive complexes, and is required for transcriptional activation by a variety of transcription factors	ASD, ID
Ubr3	Ubiquitin-Protein Ligase E3-Alpha-3: targets proteins for degradation by ubiquinitination, may be involved in chromatin regulation and transcriptional silencing (by similarity)	ASD

Table 2-1. Neurodevelopmental disorder candidate genes screened for mRNA dysregulation following chronic exposure to BDE-47 or its hydroxylated metabolites; abbreviations: ASD- autism spectrum disorder, ID- intellectual disability, SCZ- schizophrenia, HD- Huntington's disease

same 15 genes after exposure to a range of 6-OH concentrations over the various time points (**Fig. 5**). Dysregulation of mRNA levels by 6-OH was found to be dose-dependent, with transcripts of a few genes exhibiting significant changes at concentrations as low as 10nM (*Arid1b*, *Tbl1xr1*, and *Adnp*, data not shown). Of particular interest are *Arid1b* and *Smarcc2*, both of which code for subunits of the BAF chromatin remodeling complex (Ronan *et al.* 2013), as well as *Shank3*, a high confidence autism candidate gene whose protein product is involved in the organization of the postsynaptic density (Uchino and Waga 2013). In our screen, the high confidence autism candidate gene *Shank3* was significantly dysregulated by 6-OH in an opposing manner depending on the time of exposure (**Fig. 4, 5**). Together, these screens demonstrate dysregulation of mRNA levels of several NDD candidate genes by chronic 6-OH exposure in a mammalian model, and further validate the dose- and time-of-exposure dependence of 6-OH toxicity. They also provide many potential novel targets for further investigation.

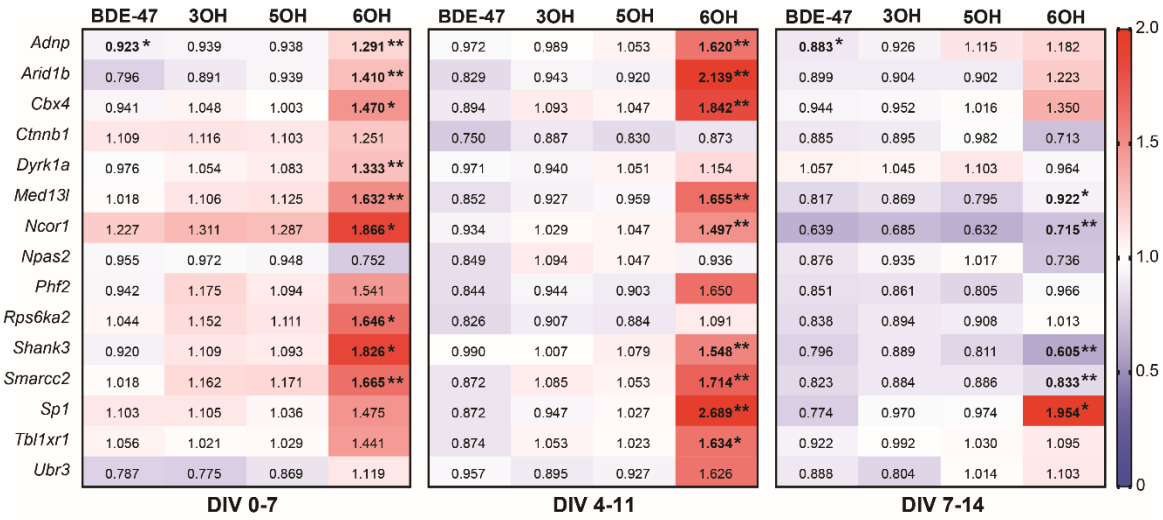


Figure 2-4: Exposure to BDE-47 and its hydroxylated metabolites dysregulates mRNA levels of neurodevelopmental disorder candidate genes. 15 neurodevelopmental disorder-associated genes screened for changes in mRNA abundance by qRT-PCR following exposures to 1μM BDE-47 or one of its hydroxylated metabolites for periods indicated below each heatmap. Each row depicts changes in mRNA level for a gene while each column reflects exposure to a different compound. Results are displayed as fold change relative to control. Cells with a fold change above two have saturated coloring. * $P < 0.05$, ** $P < 0.01$. N=3–5

While changes in mRNA levels following 6-OH exposure can be informative of altered cellular states, they do not necessarily indicate direct effects on the transcription of target genes. This is due to mRNA abundance being a function of the rate of transcription as well as of transcript decay, which can vary greatly (Garneau *et al.* 2007; Schoenberg and Maquat 2012). To verify if 6-OH-induced changes to mRNA levels were due to a direct effect on the transcription of target genes, we designed primers to detect pre-mRNAs, the short-lived, direct product of transcription by RNA polymerase II. We focused on genes strongly affected in differentiating neurons (cultures with exposure starting on DIV0) and measured

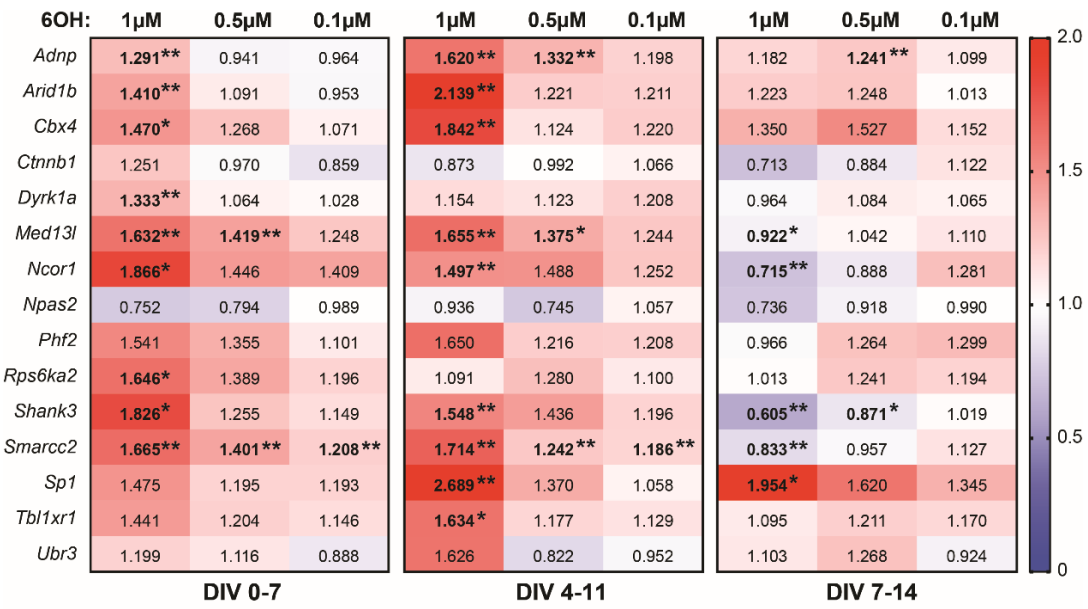


Figure 2-5: Dysregulation of neurodevelopmental disorder candidate gene mRNA levels by 6OH is dose- and time-of-exposure-dependent. 15 neurodevelopmental disorder-associated genes screened for changes in mRNA abundance by qRT-PCR following exposures to various concentrations of 6OH for periods indicated below each heatmap. Each row depicts changes in mRNA level for a gene while each column reflects exposure to a different concentration of 6OH. Results are displayed as fold change relative to control. Cells with a fold change above two have saturated coloring. * P < 0.05, ** P < 0.01. N=3–4

their respective pre-mRNA levels at time points within that window of exposure, where early neuronal differentiation is occurring (DIV0-5). Interestingly, as revealed by MTT assays and unlike what is seen in differentiated neurons (**Fig. 2C**), 1μM 6-OH did not negatively impact cell viability by DIV3 (**Fig. 6A**). In these cells, as revealed by pre-mRNA assays, 6-OH exposure impacted most genes only at the mRNA level, perhaps via indirect mechanisms that influence mRNA half-lives. However, expression profiles of three of the tested genes were found to be significantly dysregulated at the pre-mRNA level – intriguingly, all of which encode BAF complex subunits or interacting proteins (*Smarcc2, Arid1b, and Adnp*). (**Fig. 6B**).

2.4.5 6-OH-BDE-47 dysregulates expression of Smarcc2 (BAF170) and other BAF complex subunits

One of the genes exhibiting 6-OH-induced changes in pre-mRNA level, *Smarcc2,* codes for BAF170, a core subunit of the mammalian SWI/SNF (SWItch/Sucrose Non-Fermentable)-like BAF complex. BAF complexes are ATP-dependent chromatin remodelers that play important and fundamental roles in driving neurodevelopmental processes(Staahl and Crabtree 2013). BAF170 is thought to act as a scaffolding subunit that is critical for the stability of these

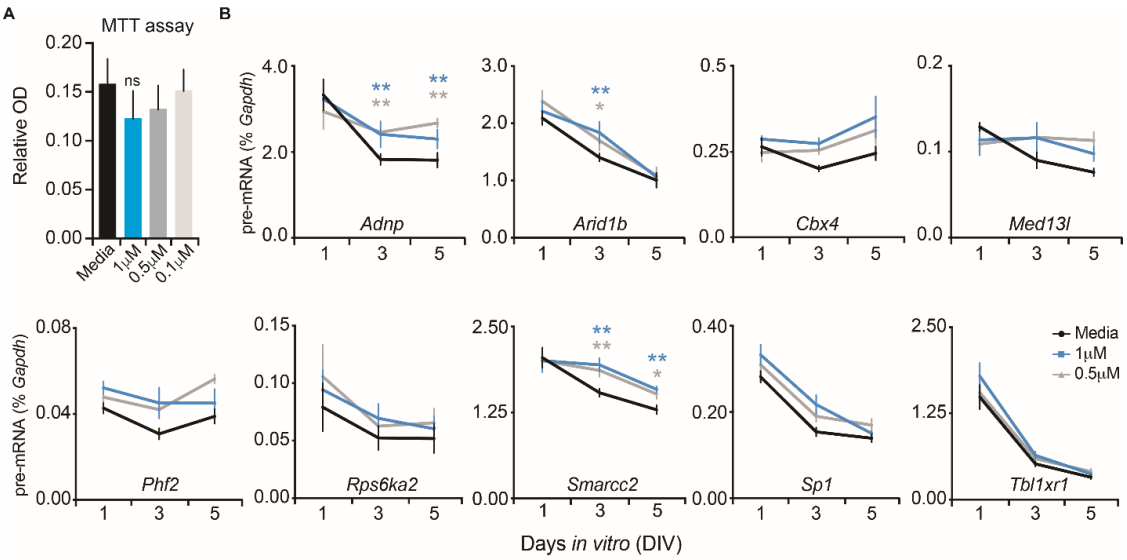


Figure 2-6: Exposure to 6OH dysregulates pre-mRNA levels of a subset of genes affected at the mRNA level. (A) Assessment of cell viability following 3 days of exposure to 1μM 6OH. Viability was assessed colorimetrically by an MTT assay. N=3. (B) A subset of neurodevelopmental disorder associated genes found to have dysregulated mRNA levels following 6OH exposure were screened for changes in pre-mRNA abundance which better reflect effects on transcription of a given gene. Time points within the earliest exposure period (DIV 0-7) were chosen to more clearly observe direct effects of 6OH toxicity. *P < 0.05, **P < 0.01. N=3–7

complexes (Narayanan *et al.* 2015). Therefore, to test the effect of 6-OH on expression of other BAF subunits, we screened mRNA levels of other known subunits of the neuronal precursor and neuron specific BAF complexes (npBAF/nBAF) and found significant upregulation of several additional subunits, namely, Brm, Brg1, BAF60a, BAF53a, and BAF47 (**Fig. 7A**). Focusing on BAF170, we then sought to confirm 6-OH-induced dysregulation of this major BAF subunit at the protein level by Western blotting. Here, to our bewilderment, we encountered two opposing outcomes with almost equal frequency across replicates. In some trials, chronic exposure of differentiating neurons to 0.1-1μM 6-OH downregulated BAF170 protein levels. In others, BAF170 protein levels were upregulated by the same treatment (**Fig. 7B**). Initially baffling, such vacillating outcomes may be explained if expression of BAF170 oscillates during neuronal differentiation and 6-OH exposure shifts the oscillation frame (**Fig. 7B**). This hypothesis is supported by previous work that found the expression of BAF170 to be very dynamic across mouse corticogenesis (Tuoc *et al.* 2013) and biphasic in H1 embryonic stem cells (Wade *et al.* 2016). To test this possibility, we collected samples at six-hour intervals after plating for the first 48 hours and then assessed BAF170 protein levels by western blotting. As shown in **Fig. 7C** (quantified in **Fig. 7D**), BAF170 levels were found to oscillate with time. Next, we attempted to quantify such oscillation with and without 6-OH exposure. To our

surprise, these oscillations varied greatly between biological Ns, occluding any statistical analysis after averaging data from multiple trials. Despite such variations, 6-OH treatment consistently altered the oscillatory pattern in every N (5 attempted; three displayed in **Fig. 7E**), providing support to our 'altered BAF170 oscillation' hypothesis (**Fig. 7B**).

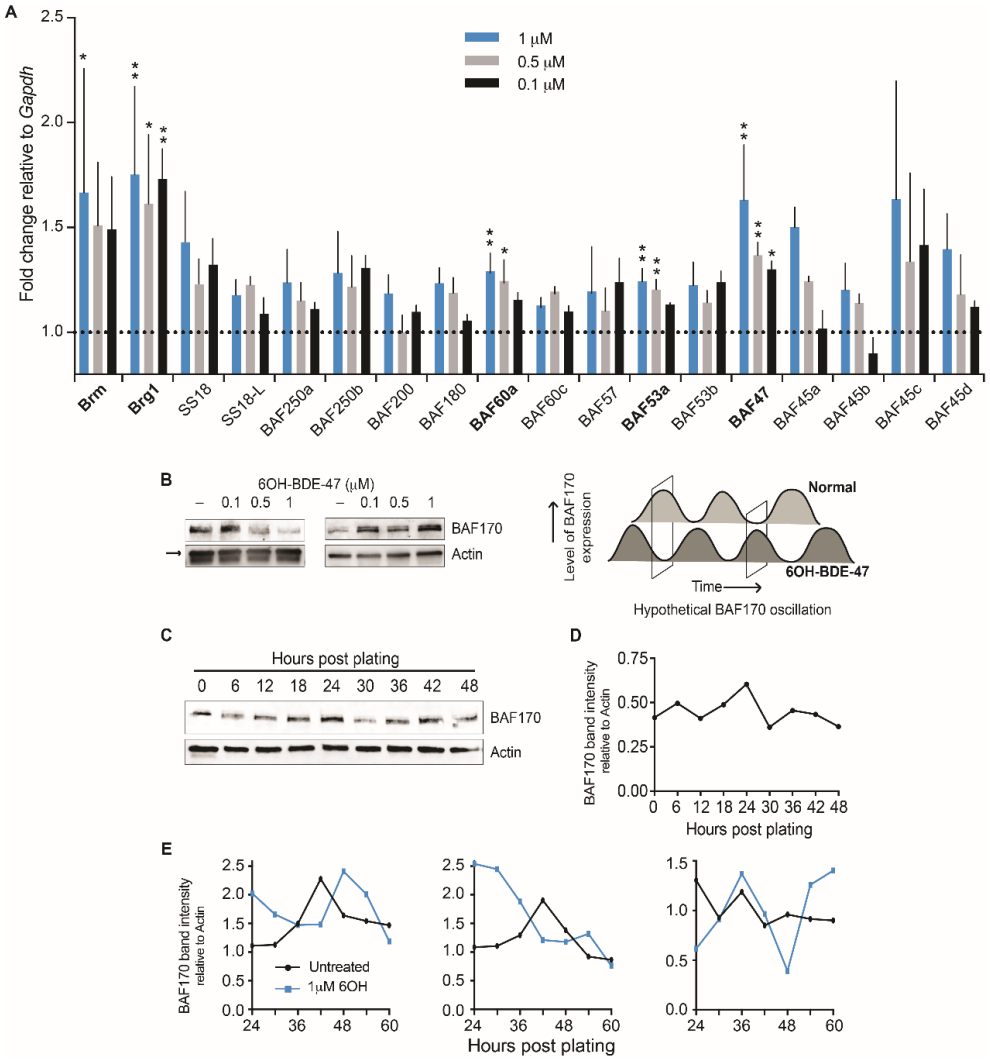


Figure 2-7: BAF170 protein levels and mRNA levels of BAF complex subunits are dysregulated by 6OH exposure. (A) Abundance of various BAF protein mRNAs estimated by qRT-PCR following exposure to various concentrations of 6OH from DIV 0-7, N=3–4. (B) Top: BAF170 protein levels observed by western blotting following 48 h of exposure to various concentrations of 6OH. N=6. Bottom: opposing outcomes can be explained if BAF170 is expressed in an oscillatory manner and 6OH exposures disrupt regulatory mechanisms shaping the temporal expression patterns. (C) Time-course western blot for BAF170 with samples collected every 6 h after plating for the first 48 h. (D) Quantification of BAF170 band intensities from blot shown in (C) as an example and evidence of BAF170 oscillation in normal cells. (E) Quantification of similar time-course experiments as in (C), with collections every 6 h starting 24 h after plating. Note the inherent variation in oscillatory pattern. Cells were either untreated or exposed to 1 μM 6OH at the time of plating. N=4 (3 represented) *P < 0.05, **P < 0.01. Note: Because of inherent variation in the oscillatory pattern, averaging these data sets obscures the phenotype and is therefore not attempted.

2.4.6 Role of BAF170 in neuronal maturation and activity induced Arc transcription

The BAF170 protein data above, alongside its altered pre-mRNA and mRNA levels, and the observed alterations to mRNA levels of other BAF subunits, indicate that 6-OH exposure likely influences BAF complex composition/stability and, therefore, potentially impacts BAF-dependent functions during neuronal differentiation, including chromatin remodeling and regulation of gene transcription.

Finally, to directly study effects of BAF170 dysregulation on functional maturation of neurons, as would be expected due to 6-OH-induced oscillation, we bi-directionally altered its levels in early stage cells (DIV 0-4) by either overexpressing BAF170 or depleting it via RNAi (lentivirus-mediated delivery of BAF170 shRNA). Both methods were validated by western blot (Fig. **8D** and **8G**). We then performed synapse-dependent and synapse-independent *Arc* induction assays. The phenotypic outcomes of these assays were unaffected by overexpression of BAF170 (**Fig. 8B and 8C**). Interestingly, depletion of BAF170 impaired *Arc* induction in the synapse-dependent Bic+4AP assay (**Fig. 8E**), but not in the synapse-independent PMA+TTX assay (**Fig. 8F**). This observation is reminiscent of the effects of lower 6-OH doses on *Arc* induction using these protocol (**Fig. 2 and 3D**). Additionally, we found that depletion of BAF170 reduces protein levels of other BAF subunits (**Fig. 8G**). These observations support the idea that BAF170 acts as a scaffolding component in BAF complexes and indicates that dysregulation of BAF170 may effectively alter BAF complex stability and composition (Narayanan *et al.* 2015) (**Fig. 7A**). Interestingly, BAF155 protein levels did not decrease while other subunits, namely BAF60a and BAF47 whose mRNA levels were significantly upregulated following 6-OH exposure (**Fig. 7A**), exhibited reduced protein levels following loss of BAF170 by RNAi (**Fig. 8G**).

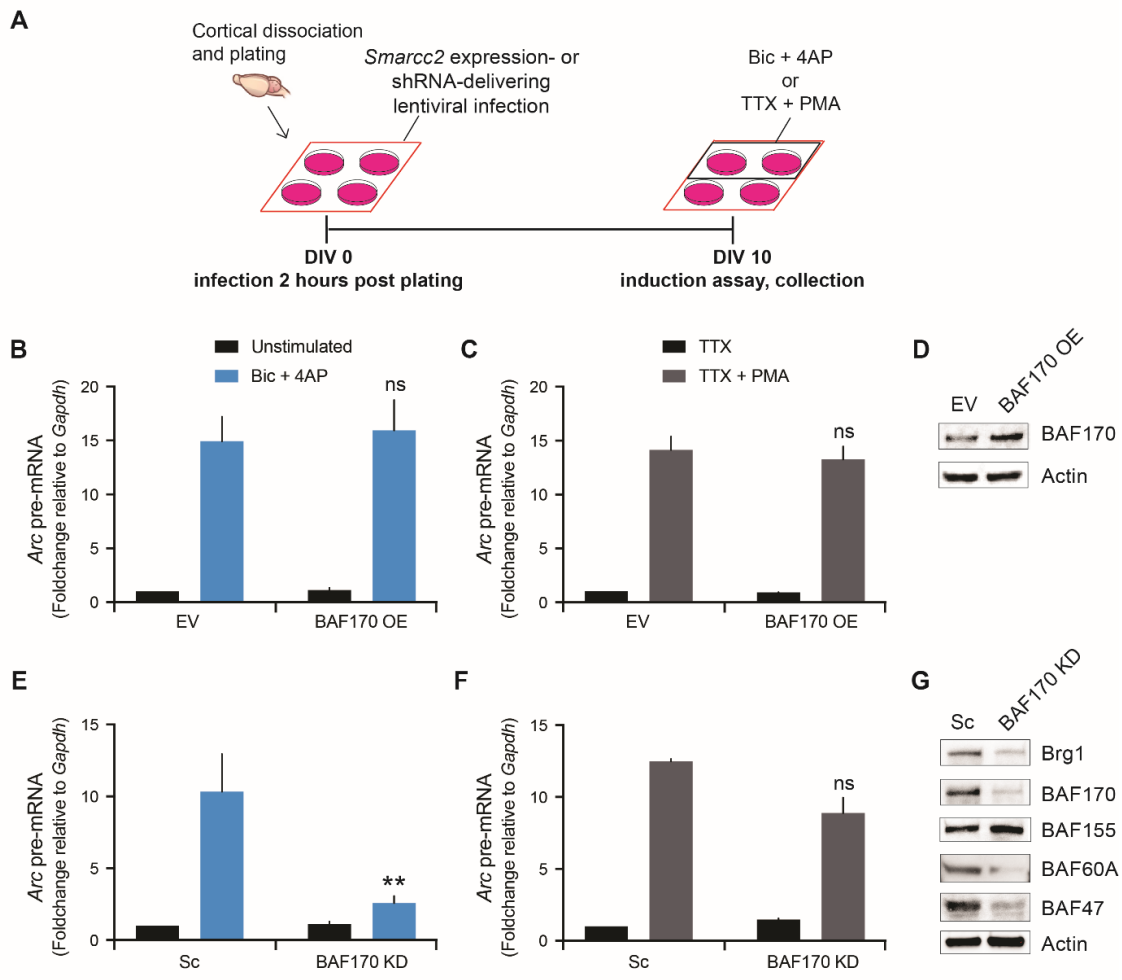


Figure 2-8: BAF170 contributes to the stability of BAF subunits and proper circuit formation, but is not required for MAPK stimulated Arc transcription. (A) Depiction of the timing of lentiviral mediated manipulation of BAF170 levels and subsequent activity induced Arc induction assays. (B, C) Arc induction following Bic-4AP or TTX-PMA stimulation in cells overexpressing BAF170, N=3. (D) Representative western blot validating BAF170 overexpression construct. (E, F) Arc induction following Bic-4AP or TTX-PMA stimulation in cells depleted of BAF170, N=3. (G) Representative western blots demonstrating the effect of BAF170 knockdown on protein levels of other BAF complex subunits, N=3. **P < 0.01. ns = non-significant

Taken together, our data suggest that BAF170 depletion, not its overexpression, likely impairs neuronal maturation via synapse-dependent mechanisms, but not extra-synaptic components of activity-induced gene transcription. As effects of BAF170-KD are highly evocative of the effects of 6-OH exposure, we propose that environmentally-relevant doses of 6-OH may act via dysregulation of the BAF complex, especially BAF170, to interfere with neuronal maturation and function.

2.5 Discussion

In this chapter, I have demonstrated several novel aspects of PBDE neurotoxicity. Initially, I show the effects of chronic nanomolar range exposures to BDE-47 and its hydroxylated metabolites on neuronal activity-induced gene transcription and functional maturation of primary embryonic rat cortical neurons. I then demonstrate how similar exposures impact transcription of NDD candidate genes by quantification of mRNA and pre-mRNA levels. One of the strongest candidates from our screen, *Smarcc2*, then prompted us to focus on the effects of 6-OH exposure on BAF170 expression and, preliminarily, on BAF170-related regulation of neuronal maturation by measuring levels of activity-induced *Arc* expression. Together, these results indicate that exposure to the hydroxylated BDE-47 metabolite 6-OH strongly influences functional maturation of neurons, potentially independent of thyroid hormone disruption mechanisms– perhaps instead exerting effects mediated in part at the level of epigenetic regulation via disruption of BAF chromatin remodeling complexes.

Our utilization of the activity-induced transcription of an immediate early gene (*Arc*) as a readout of functional neuronal maturation is, to the best of our knowledge, a novel approach in assessing the neurotoxicity of PBDEs. Immediate early gene induction is a strong tool to this end as it relies on several critical cellular mechanisms that are established across neuronal maturation, ranging from proper synaptic function to nuclear regulation of gene transcription (**Fig. 3A**), allowing us to broadly assess the extent of PBDE-induced effects on functional neuronal maturation. Further, inducing *Arc* expression both synaptically (Bic+4AP) and extra-synaptically (TTX+PMA) allowed us to hone into where these neurotoxic effects are being exerted. These assays reveal that 6-OH-BDE-47 interferes with neuronal maturation likely at the synaptic level.

As mentioned previously, the range of PBDE doses used in this chapter were estimated based on prior work done in mammalian models to approximate relevant concentrations observed in the brains of exposed animals. To determine this range, an exhaustive review was conducted of literature reporting levels of BDE-47 accumulation in rodent brain following either chronic or single dose administration (Reistad *et al.* 2006; Staskal *et al.* 2006; Zhang *et al.* 2008; Ta *et al.* 2011; Koenig *et al.* 2012; Rasinger *et al.* 2014; Costa *et al.* 2015). After converting the commonly reported unit of ng/g of toxin to nmol/L (converting from lipid concentration to molarity), the average concentration of BDE-47 was determined to be 597nM. Studies investigating the *in vitro* effects of PBDEs have often been conducted using concentrations in the low to mid micromolar range. Summarizing human studies, the USEPA's 2010 report assessing the environmental prevalence of PBDEs estimated the body burden of total PBDEs in the general population to be 30-100 ng/g (approximately 60 to 200nM), with toddlers tending to have higher body burdens compared to older children and adults. Importantly, BDE-47 alone was estimated to account for 50% of total

PBDE concentrations (U.S. Environmental Protection Agency (EPA) 2010). It has also been reported that hydroxylated metabolites of PBDEs accumulate in human serum and are nearly as abundant (approximately 45% of total detectable PBDEs) as parent PBDE compounds in human fetal blood samples collected in the United States (Athanasiadou *et al.* 2008; Qiu *et al.* 2009). Considering the reported brain accumulation levels of BDE-47 in rodents, concentrations commonly used for *in vitro* studies to date, and the prevalence of BDE-47 and its hydroxylated metabolites in humans, we used a range of 100-1,000nM BDE-47 and its hydroxylated metabolites for our studies. This range spans reported accumulated concentrations in rodent brain, is low relative to previous *in vitro* studies, and is on the same order of magnitude as reported human levels of PBDEs and their metabolites.

It has been previously demonstrated that micromolar BDE-47 exposures decrease both neuronal and oligodendrocyte differentiation in human neural progenitor cells (Schreiber *et al.* 2010). Another study found that BDE-47 and 6-OH decrease neuronal and oligodendrocyte differentiation of mouse adult neural stem cells (aNSC), with 6-OH producing an effect following nanomolar exposures, at concentrations approximately 20 times lower than its parent compound (Li *et al.* 2013). Using activity-induced gene transcription assays as a readout in cultured embryonic rat cortical neurons, we found that chronic nanomolar exposure to 6-OH disrupts functional maturation. These effects were found to be independent of 6-OH cytotoxicity or alteration of differentiation for 0.5μM exposures. Many of the observed effects were found to be dose-dependent whereas transcriptional profiles (**Fig. 4 and 5**) were additionally sensitive to time of exposure.

Additionally, these functional effects appear to be mediated at both synaptic and extra-synaptic levels, as evidenced by decreases in *Arc* induction following Bic+4AP and TTX+PMA treatments. Effects on IEG transcription by both assays indicate that 6-OH, at higher doses, may act to compromise cell viability, disrupt mature synapse formation, as well as interfere with cellular mechanisms at or downstream of the MAPK pathway that regulate activity-induced *Arc* transcription (**Fig. 2 and 3**). Interestingly, addition of excess levels of the thyroid hormone triiodothyronine (T3) did not produce any phenotypic rescue (as seen previously for BDE-47 induced impairment of neuronal migration (Schreiber *et al.* 2010)) of the observed attenuation of *Arc* induction. This advocates for additional modes of PBDE toxicity, perhaps acting alongside disruption of thyroid hormone signaling. However, we also note that 1μM 6-OH-induced cytotoxicity partially confounds interpretation of this attempted T3 rescue, as reduction of overall cell viability may obscure potential reversal of the effects of 6-OH exposure by T3. To explore alternate modes of PBDE toxicity within a context of the relation of PBDE exposure to NDDs, we screened 15 NDD candidate genes, many of which are epigenetic regulators (**Table 1**), for changes in mRNA level. Subsequently, we found that, while exposure to the parent compound and its other hydroxylated

metabolites significantly impacted only a few of these targets, 6-OH dysregulated mRNA levels of many of the screened genes. These included *Shanks3,* which encodes an important synaptic protein, supporting our inference related to detrimental synaptic effects of persistent 6-OH exposure.

Of the genes dysregulated by 6-OH exposure, a smaller subset additionally exhibited altered levels of pre-mRNA, indicating a direct effect of 6-OH on transcription of these genes. Surprisingly, each of the genes whose pre-mRNA was found to be significantly dysregulated encode BAF chromatin remodeling complex subunits or interacting proteins (*Adnp*, *Arid1b*, *Smarcc2*). One of these genes, *Smarcc2*, encodes a critical core subunit known to contribute to overall BAF complex stability and composition, BAF170. Protein levels of BAF170 were subsequently found to be dysregulated by 6-OH exposure, though in a complex oscillatory manner that remains to be fully characterized. It may be the case that as lack of BAF170, but not overabundance, is detrimental for neurons (as we have shown in Figure 8), the cells are responding to repeated periods of lack of sufficient BAF170 by upregulating *Smarcc2*, while protein levels vary due to post-translational regulation.

BAF170 is a striking candidate whose dysregulation may contribute to the observed effects of 6-OH toxicity, owing to its critical contribution to BAF complex function. It has been shown to regulate the stability of BAF chromatin remodeling complexes by acting as a scaffolding subunit and preventing degradation of other BAF proteins. Our results support this notion and demonstrate a reduction of protein levels of several BAF subunits upon BAF170-KD (**Fig. 8G**), an effect previously only seen with removal of both BAF170 and another interchangeable complex subunit, BAF155 (Narayanan *et al.* 2015). This may indicate that BAF170 plays a larger role in stabilizing BAF complexes or specific subunits during the periods of neuronal maturation investigated in this chapter. This stabilizing aspect of BAF170 function, making it critical for BAF complex function as a whole, is intriguing as BAF complexes have well known roles in neuronal differentiation and maturation (Ronan *et al.* 2013), and are thought to contribute to regulating activity-induced gene transcription (Stunkard and Ghosh 2008; Zhang *et al.* 2016; Lyons and West 2011). As such, periods of 6-OH induced lack of BAF170 during early stages where differentiation and maturation taking place in our cultures may compromise the functional maturation of cells, leading to the observed decrease in *Arc* expression when assayed at later time-points.

Accordingly, we have shown that reduction of BAF170 levels by RNAi, but not its overexpression, negatively impacts neuronal maturation using *Arc* induction as a readout, recapitulating the effect of exposure to 0.5μM 6-OH following Bic+4AP treatment. The lack of effect seen after BAF170 depletion on extra-synaptically induced *Arc* (TTX-PMA protocol), as was the case with lower doses of 6-OH in Figure 3C, indicates that BAF170-containing BAF complexes may not be necessary for nuclear regulation of activity-induced *Arc* transcription within the

first 15 minutes after the onset of activity, but most likely contribute to synapse maturation and function via transcriptional regulation of other genes. These findings are consistent with recent evidence supporting the idea that BAF complexes are crucial for synapse formation and maturation during early neuronal development (Zhang *et al.* 2016). The same study implicated a role of Brg1, a core BAF complex ATPase, in activity-induced gene transcription six hours after onset of activity. However, as our data indicate, BAF170-containing BAF complexes are not specifically required for *Arc* induction at early time points if activity is induced extra-synaptically, likely owing to its poised transcriptional status (Saha *et al.* 2011). This lack of effect following BAF170 depletion and TTX+PMA treatment may be gene-specific as various IEGs are regulated in differing manners (Lyons and West 2011; Flavell and Greenberg 2008; Saha *et al.* 2011).

While our current work potentially opens new avenues to explore the effects imposed by hydroxylated BDE-47 metabolites on epigenetic regulation during neuronal maturation and activity-induced gene expression, there are limitations to the experimental work in this chapter. Although we have demonstrated that exposure to 6-OH has a strong effect on BAF170 expression, from the pre-mRNA to protein level, the effect is of a complex nature, whose mechanistic basis we do not yet fully understand. Consistent with our findings, the multiphasic expression pattern of BAF170 has been previously demonstrated during corticogenesis in mice (Tuoc *et al.* 2013) and in H1 embryonic stem cells (Wade *et al.* 2016), but further investigation will be required to fully characterize the expression profile of BAF170 and regulatory mechanisms that shape it. Additionally, like much PBDE research, we have investigated the effects of exposure to a single toxin at a time, while the developing human nervous system is realistically exposed to complex mixtures of pollutants. Some studies have been completed demonstrating effects of coexposures to environmental pollutants both *in vitro* and *in vivo* using combinations of PBDE congeners, other toxins including polychlorinated biphenyls (PCBs), and commercially available mixtures such as DE-71 (Pellacani *et al.* 2009; Tagliaferri *et al.* 2010; Miller *et al.* 2012; He *et al.* 2011). More such studies will be needed to address the effects resulting from environmentally relevant mixtures of compounds, especially with concern to their metabolites.

Finally, our results show that nanomolar range exposures to the BDE-47 hydroxylated metabolite 6-OH strongly impact several aspects of neuronal maturation and function and that these effects are likely, at least in part, mediated by disruption of BAF chromatin remodeling complexes via dysregulation of BAF170 expression. Going forward, it will be important to fully characterize the regulatory mechanisms governing the intricate expression of BAF170 to discern precisely how the observed PBDE-induced dysregulation is produced. Additional investigation of the underlying mechanisms that mediate the neurotoxic effects of PBDE at the level of epigenetic regulation may lead to a

better understanding of how exposure to these compounds leads to developmental complications and NDDs as well as to further characterization of fundamental neuroepigenetic processes.

Chapter 3: Specific ortho-hydroxylated brominated ethers inhibit neuronal MEK-ERK signaling and disrupt neurodevelopmental processes

In preparation: Robert G. Poston, Lillian Murphy, Ayna Rejepova, Mina Ghaninejad-Esfahani, Joshua Segales, Kimberly Mulligan, Ramendra N. Saha

3.1 Abstract

The developing nervous system is remarkably sensitive to environmental signals, including disruptive toxins, such as Polybrominated diphenyl ethers (PBDEs). PBDEs are an environmentally pervasive class of brominated flame retardants whose association with behavioral deficits in humans remains mechanistically unresolved. Using dissociated cortical neurons from embryonic rats (*Rattus norvegicus*), we found that chronic exposure to 6-OH-BDE-47, one of the most prevalent hydroxylated PBDE metabolites, suppressed both spontaneous and evoked electrical activity. Based on our previous work on MEK-ERK biology and our observation that 6-OH-BDE-47 shares key structural similarities to Type-III non-ATP competitive MAP kinase MEK1 inhibitors, we hypothesized that certain hydroxylated PBDEs mediate neurotoxicity, at least in part, by inhibiting the MEK-ERK axis of MAPK signal transduction. We tested this hypothesis on three platforms: 1) *in silico*, where modeling ligand-protein docking revealed that the MEK1 inhibitor (PD-0325901) and 6-OH-BDE-47 docks to MEK1 at the same binding site, 2) *in vitro* in dissociated neurons, where MEK1 inhibitor and 6-OH-BDE-47 — but not other hydroxylated BDE metabolites — similarly impaired MEK1-dependent phosphorylation of ERK1/2 and activity-induced transcription of a neuronal immediate early gene, and 3) *in vivo* in *Drosophila melanogaster*, where developmental exposure to either the MEK1 inhibitor or 6-OH-BDE-47 resulted in offspring displaying similar increased frequency of mushroom body β-lobe midline crossing, a metric of axonal guidance. Taken together, our data supports a novel toxicity mechanism for certain ortho-hydroxylated PBDE metabolites, which can inhibit MEK-ERK signaling and cause disruptions of critical neurodevelopmental processes, including spontaneous and evoked electrical activity, synaptic functions, and axonal guidance.

3.2 Introduction

Previously, we utilized the ability of neurons to induce gene transcription in response to elevated network activity as a tool for assessing developmental PBDE toxicity (Poston *et al.* 2018). Specifically, we assayed levels of the neuron-specific activity-induced immediate early gene (IEG) *Arc* after exposing embryonic cortical neurons across various stages of differentiation and maturation. Our rationale for employing this endpoint was that *Arc* induction relies on multiple levels of regulation, from electrical activity and synaptic function, to intracellular signaling (particularly Ca^{2+}-dependent mechanisms), to nuclear regulation of gene expression — all of which are critical processes intertwined in regulating neurodevelopment (Spitzer 2006). These processes are largely hardwired by genetic programs but are also modulated by environmental cues. Exposure to toxins like PBDEs represent an environmental challenge that may dysregulate these processes at multiple levels, the cumulative effects of which could be detected as altered transcriptional responses. Results from the previous chapter showed that chronic exposures to a hydroxylated metabolite of BDE-47, 6-OH-BDE-47 (6-OH), dysregulate basal and activity-dependent neuronal transcription. Among those findings were data which strongly indicated that the attenuated transcriptional response following 6-OH exposure was mediated by synaptic dysfunction, and that 6-OH is capable of inhibiting intracellular signaling. Either could explain the attenuated transcription of *Arc*, as its activity-induced transcription relies on synaptic activity as well as signaling via the Mitogen Activated Protein Kinase (MAPK) cascade, one of the major signaling pathways that is important for neuronal plasticity (Thomas and Huganir 2004) and, as we have recently shown, is necessary for *Arc* induction (Tyssowski *et al.* 2018).

In this chapter, I propose and test a novel mechanism of PBDE metabolite toxicity: via inhibition of a kinase in the MAPK signaling pathway. The notion that a PBDE metabolite could have the capacity to inhibit MAPK signaling was of particular interest to us for several reasons. The MAPK pathway is a deeply evolutionary conserved intracellular signaling mechanism that supports diverse processes in both prokaryotes and eukaryotes (Pérez *et al.* 2008; Pereira *et al.* 2011; Widmann *et al.* 1999). In prokaryotes, PBDE inhibition of MAPK-related serine/threonine kinases could explain the natural biogenesis of these compounds, known to have antimicrobial activity and to be produced by bacteria in several marine contexts, including sponge-cyanobacterial symbiotic relationships (Sharma and Burkholder 1967; Burkholder and Sharma 1969; Sharma *et al.* 1970; Unson *et al.* 1994; Malmvärn *et al.* 2005; Malmvärn *et al.* 2008; Agarwal *et al.* 2014; Agarwal *et al.* 2017). In eukaryotes, the most well-known role of MAPK is regulation of cell growth, division, and differentiation in many cell types, including stem cells in the developing nervous system (Zhang and Lie 2002; Li *et al.* 2006; Rhim *et al.* 2016). It is also known to regulate axonal

guidance and growth which has been demonstrated in multiple species (Bülow *et al.* 2004; Tang and Kalil 2005; Xing *et al.* 2016; Igarashi and Okuda 2019). Additionally, genetic syndromes involving the dysregulation of the MAPK pathway, known as the 'RASopathies,' are known to be involved in certain NDDs (Rauen 2013; Millan 2013). In mature neurons – post-mitotic non-dividing cells – the pathway regulates synaptogenesis (Huang and Reichardt 2001; Giachello *et al.* 2010), synaptic plasticity (Martin *et al.* 1997), activity-induced gene expression (Lyons and West 2011; Tyssowski *et al.* 2018), and generally, communicates information to a large number of cellular substrates. These roles in neural cells make MAPK signaling a concerning target for environmental disruption. However, the MAPK pathway remains largely unexplored as a target for pollutants.

With these factors in mind, we sought to expand and clarify our previous findings with the studies reported here. Here, we show that chronic 6-OH exposure suppresses neuronal electrical activity and that acute exposures can inhibit MEK-ERK signaling, a crucial signaling cascade that couples neuronal activity with activity-dependent processes including gene transcription. We also report that *in vivo* exposures to 6-OH mimic exposure to the MEK inhibitor PD0325901 and similarly alter axonal guidance in the mushroom bodies of *D. melanogaster*. These new findings corroborate our earlier work and further demonstrate the ability of hydroxylated PBDE metabolites to disrupt neurodevelopmental processes via a novel mechanism.

3.3 Materials and Methods

3.3.1 Primary neuronal culture and cell treatment

Primary cultures of cortical neurons were prepared from embryonic day 18 Sprague Dawley rats (Charles River) (UC Merced IACUC approval: AUP#16-0004). Isolated cortical hemispheres were collected in magnesium and calcium-containing HBSS and mechanically dissociated by pipetting with a fire-polished Pasteur pipet following a short digestion with StemPro® Accutase® (LifeTech, A1110501). Dissociated cortical neurons were then pelleted by centrifugation, resuspended in plating media, counted using trypan blue and a TC20™ automated cell counter (BIO-RAD, #1450102), and subsequently plated by addition to dishes containing pre-warmed Neurobasal medium (Invitrogen) supplemented with 25 μM glutamate (Sigma-Aldrich), 0.5 mM L-glutamine (Sigma-Aldrich), and 2% NS21 supplement. Cells were maintained in the same medium without glutamate, exchanging half the conditioned media with fresh every 3-4 days. NS21 was prepared in the laboratory as previously described (Chen *et al.* 2008). Neurons were routinely used for various assays between 10–14 days *in vitro*. To induce MEK-ERK signaling and gene transcription using synaptic activity, neurons were co-treated with 50 or 5μM Bicuculline (Sigma-Aldrich) with or without 75μM 4-Aminopyridine (Acros Organics). To induce gene

transcription extra-synaptically, we blocked activity with 1μM TTX (Calbiochem) and induced the MAPK pathways via PKC with 1μM-1nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich). BDE-47 and hydroxylated BDE metabolites used to treat cultures were obtained from AccuStandard (BDE-047N, HBDE-4003N, HBDE-4004N, HBDE-4005N) and initially dissolved in dimethylsulfoxide (DMSO) at 2mM stock concentrations. Final treatment concentrations were produced at the time of treatment by dilution in culture medium. PD0325901 was obtained from AdipoGen (SYN-1059) and dissolved in DMSO at 5mM and similarly diluted at the time of treatment.

3.3.2 Microelectrode array recording and data processing

Neurons from the preparations described above were plated on poly-L-lysine/laminin coated MEAs (60MEA200/30-Ti, Multi Channel Systems (MCS), Reutlingen, Germany) in 500 μl of the NS21-supplemented Neurobasal plating medium described above. When cells were fed, again every 3-4 days, approximately half the media was replaced with NS21-supplemented BrainPhys feeding media (StemCell). This was done to promote optimal conditions for neuronal firing which has been shown to be enhanced in BrainPhys media (Bardy *et al.* 2015). Recordings were made by placing arrays in an amplifier headstage that interfaces with MCS provided Multi Channel Experimenter software. Sampling was conducted at 10-20kHz in 3-minute sessions at room temperature (arrays were covered to prevent contamination). Recording were post-processed in Multi Channel Analyzer with a high-pass 1st order Butterworth filter with 100hz cutoff prior to quantification of spike number, determined by an automatic threshold estimator set to 6-8 standard deviations from the baseline signal. To quantify burst properties, the Multi Channel Analyzer burst detection tool was used with the following settings: max. interval to start burst, 25ms; max. interval to end burst, 250ms; min. interval between bursts, 1500ms; min duration of burst, 50ms; min. number of spikes in burst, 5. Raster images from example recordings were generated in NeuroExplorer (NexTechnologies, Herdon, VA).

3.3.3 Ligand-Protein docking simulations

For ligand-protein docking simulations, we used AutoDock Vina, an open-source molecular docking program made available through the Scripps Research Institute (Trott and Olson 2010). Initially, blind-docking of 6-OH-BDE-47 and PD0325901 was conducted with large areas of a published crystal structure of human MEK1 (PDB ID: 3EQI, Fischmann et al. 2009). Subsequent simulations searching a smaller grid-space centered around the catalytic binding domain were then conducted. The simulations were run with standard parameters for a rigid receptor and ligands with rotable bonds (Forli *et al.* 2016). The binding structures were evaluated based on comparison to the well characterized binding modes of allosteric MEK inhibitors, the first example of which was published in 2004 (Ohren *et al.* 2004).

3.3.4 Western blotting and imaging

Neurons were lysed in ice-cold 1X RIPA buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Na- deoxycholate, 0.1% SDS, 0.1% NP-40) supplemented with 1:100 protease inhibitor cocktail (Sigma-Aldrich-Aldrich). Nuclear lysates were prepared by first washing cells with ice-cold 1X PBS, applying Plasma Membrane Lysis Buffer (PMLB) (25 mM Tris-HCl, pH 7.6, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT, 0.1% NP-40) for 1 min on ice, washing with PMLB, and then collecting in 1X RIPA buffer (pH 7.4). Lysates were sheared by sonication (low setting; three cycles on Bioruptor®), cell debris pelleted at 15,000 rpm for 5 minutes at 4°C, and clarified supernatant transferred to pre-chilled 1.5 mL microcentrifuge tube. Synaptosomal isolations were prepared using Syn-Per according to manufacturer instructions (ThermoFisher). The various cell extracts were denatured at 95°C, for 5 minutes, using either 2X-, or 4X-Laemmli sample buffer (BIO-RAD). Denatured protein samples were resolved on 4-20%- (BIO-RAD cat. no. 4568095) or 4-15%- (BIO-RAD #456-1083) Mini PROTEAN® gels in Tris/Glycine/SDS (BIO-RAD #1610772). Resolved proteins were transferred onto LF PVDF membrane, using the BIO-RAD TBT RTA kit and protocol using either 10%MeOH or 20%EtOH-containing transfer buffer (cat. no. 1704272). PVDF membranes were incubated at 4°C overnight with appropriate primary antibodies in 1X TBS-T with 1.5% BSA. Primary antibodies included the following antibodies: β–Actin (Invitrogen, AM4302), H4 (CST, 2935), pERK (CST, 4370), Synapsin-I (SySy, 106011), PSD-95 (NeuroMab, 75-028). Next day, membranes were washed three times in 1X-TBST for 5 min each, probed with either goat-anti-Mouse-647 or goat-anti-Rabbit-546 Alexa Fluor® secondary antibodies (Life Technologies) for 45 minutes at room temperature, washed three times with 1X TBS-T for 5 min each, and imaged using BIO-RAD Multiplex ChemiDoc™ Imaging System.

3.3.5 RNA extraction and gene transcription quantitation

Total RNA was isolated from cultured neurons using the illustra RNAspin Mini kit (GE Lifesciences). Specific pre-mRNAs from these total RNA samples was initially amplified by cDNA synthesis (14 cycles) using *Arc* and *Gapdh* primers overlapping an intron-exon junction and a OneStep RT-PCR kit (Qiagen, #210212). Quantitative real-time PCR (qRT-PCR) was then performed from this cDNA to quantify levels of specific transcripts using iTaq Universal Sybr Green Supermix (BioRad) and the BIO-RAD CFX Connect realtime PCR Detection System. Fold-change from control was estimated using the delta-delta Ct method.

3.3.6 Drosophila rearing & chemical exposure

Wild-type Canton-S *Drosophila* were maintained at 25°C on a standard cornmeal diet (Bloomington Drosophila Stock Center standard cornmeal medium). For oral administration of chemicals, PD0325901, BDE-47, 5-OH, and 6-OH were dissolved in dimethylsulfoxide (DMSO) and then added directly to warm food, prior to solidification. The concentration of DMSO was maintained at 0.02% for all exposures. *Drosophila* were exposed to PD at a concentration of 100nM. For all other chemicals (PBDE-47, 5-OH, and 6-OH), *Drosophila* were exposed to a concentration of 1uM.

3.3.7 Fluorescence labeling and microscopy of Drosophila whole-mount brain preparations.

Whole-mount immunostaining of adult brains was performed as described in Michel et al., 2004, except that brains were fixed for 25 minutes in 4% paraformaldehyde. Anti-Fasciclin II (FasII; monoclonal antibody ID4; Developmental Studies Hybridoma Bank, Iowa City, IA) was used at a 1:20 dilution. Alexa488-conjugated goat anti-mouse secondary (Jackson ImmunoResearch, West Grove, PA) was used at a 1:1000 dilution. Images were captured using an Olympus laser scanning confocal microscope.

3.3.8 Statistical analysis

Error bars represent standard error of the mean throughout the article, except in Fig.7D where they represent 95% confidence intervals. Statistical analyses were conducted using GraphPad Prism 7 (GraphPad software, San Diego, CA). Data were analyzed by either one or two-way ANOVA with appropriate post-hoc tests for generation of specific *P*-values– either FDR correction method or LSD. The only exception is Fig.7D where the non-parametric Mann-Whitney U test was used to compare the medians of effect score distributions for the various treatment groups. Biological replicates are indicated throughout as *N* in corresponding figure legends. Biological replicates constitute cell culture preparations from the pooled cortices of embryos from independent litters or brains of individual flies.

3.4 Results

3.4.1 Chronic 6-OH-BDE-47 exposure suppresses electrical activity in cortical neurons

We have previously shown that chronic exposure to nanomolar concentrations of 6-OH-BDE-47 (6-OH) impairs the ability of primary cortical neurons to induce transcription of the IEG *Arc* (Poston *et al.* 2018). Findings from that work indicated that the effects of the exposure were manifested at the level of synaptic functionality. To test this hypothesis directly, we utilized the same cell culture system, but grew cells on microelectrode arrays (MEAs). Cells were exposed to 500nM 6-OH from the day of plating. Spontaneous electrical activity of the networks were recorded daily **(Fig. 1A)**. There were no obvious morphological differences in cell growth. We also previously demonstrated that this exposure level does not significantly impact cell viability (Poston *et al.* 2018). Spontaneous activity was detected usually starting at 6 days *in vitro* (DIV), which is consistent with previous reports for cultured cortical neurons (Charlesworth *et al.* 2015), and continued to the end of the experiment at DIV14. Networks exposed to 6-OH displayed significant suppression of spontaneous spiking

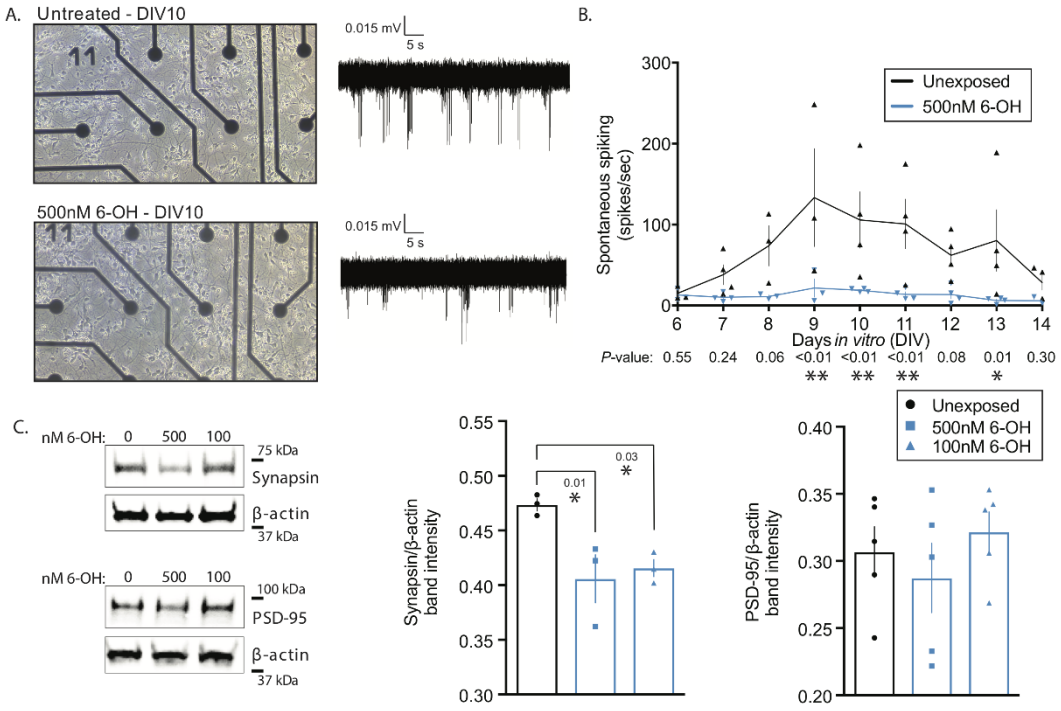


Figure 3-1. Chronic 6-OH-BDE-47 exposure suppresses spontaneous neuronal activity and alters pre-synapses. Primary rat cortical neurons were grown on MEAs in order to assess the effects of 6-OH exposure on electrical activity. (A) Examples images of neurons at DIV10 with or without exposure to 500nM 6-OH (left) example traces of recorded spontaneous activity (right) (B) Detectable activity was recorded and quantified through the first two weeks of growth in vitro, N=3-4. (C) Synaptosomes were isolated from DIV10 cultures. Pre-and post-synaptic markers were assessed by western blotting(left) densiometric quantification of blots (right), N=3-5. * indicates P-value <0.05.

across the recording period which was quantified and is summarized in **Fig. 1B**. To further clarify the synaptic nature of the exposure effects, we isolated synaptosomes from 6-OH treated cultures (DIV0-10) and evaluated levels of both pre- and post-synaptic protein markers by western blotting. We found a significant decrease in the amount of detectable Synapsin-I, but not PSD-95 **(Fig. 1C)**, indicating that 6-OH exposure likely imparts pre-synaptic dysfunction, although post-synaptic effects unrelated to PSD-95 cannot be ruled out. Together, these data support the hypothesis generated from our earlier work that chronic exposure to the BDE-47 hydroxylated metabolite, 6-OH, impairs synaptic composition and function.

Next, we studied the effects of 6-OH on induced activity in neurons. This was partly to explain one of our previous observations where activity-induced expression of the IEG *Arc* was significantly reduced in networks exposed chronically to 6-OH (Poston *et al.* 2018). To test if chronic 6-OH exposure impaired firing patterns during induced activity – which could explain impaired *Arc* transcription – we evoked activity in unexposed and 6-OH exposed networks using two different bicuculline (Bic) stimulation paradigms **(Fig. 2A)**. The first was continuous treatment with Bic, similar to the approach we previously used to induce *Arc* transcription (Poston *et al.* 2018). The other was a Bic stimulus

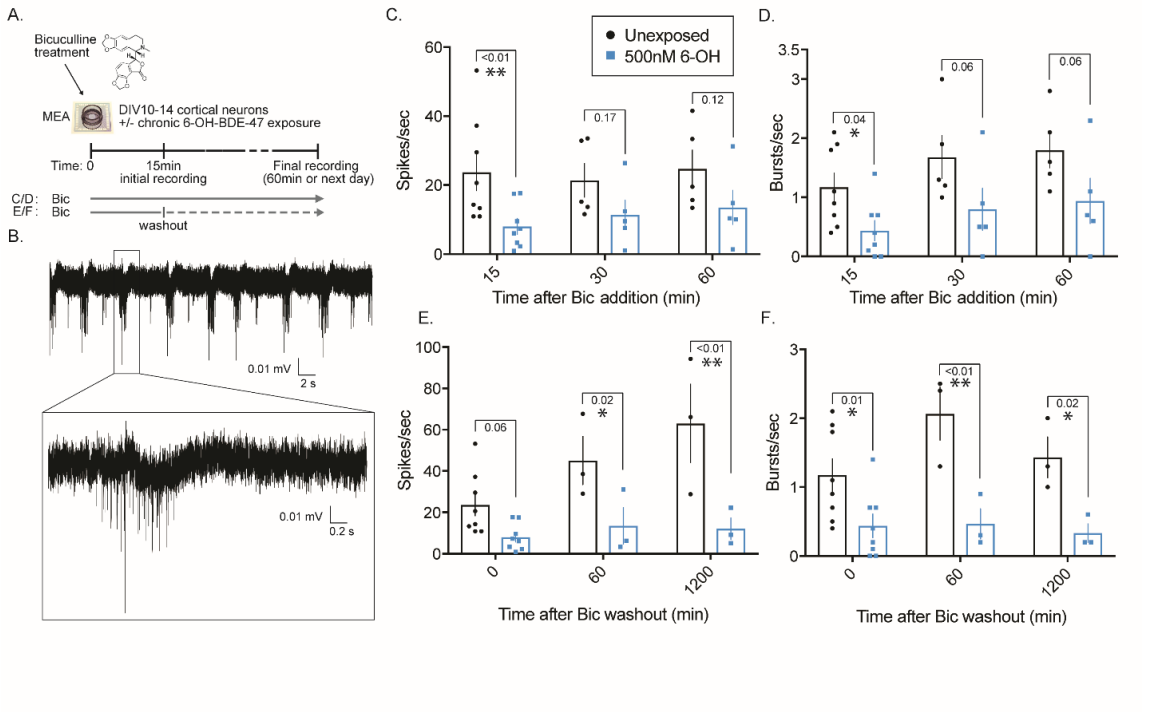


Figure 3-2. Chronic 6-OH-BDE-47 exposure impairs evoked synaptic activity. Multiple stimulus paradigms were used to assess the effects of chronic 6-OH exposure on evoked electrical activity. (A) Graphical depiction of bicuculline stimulus paradigms. (B) Example of characteristic burst activity generate with Bic stimulus. (C&D) Quantification of spikes and bursts detected under continual Bic treatment, N=5-8. (E&F) Quantification of spikes and bursts detected after brief Bic stimulation and washout, N=3-8. * indicates P-value <0.05.

followed by washout, a treatment paradigm known to produce recurrent synchronous bursting that has been previously published as a model of *in vitro* plasticity (Arnold *et al.* 2005). Both types of Bic treatments produced characteristic burst firing patterns **(Fig. 2B)** that were diminished in 6-OH treated cells (**Fig. 2C-F**). The observed reduction in activity after 15 minutes of continuous Bic treatment (**Fig. 2C, D**) directly explains our earlier finding that chronic 6-OH exposure impairs activity-induced *Arc* transcription due to reduced firing of exposed neurons. The strong effect, seen under the Bic washout paradigm (**Fig. 2E, F**), further demonstrates the compromised synaptic functionality of 6-OH treated cells.

3.4.2 6-OH-BDE-47 shares key structural features of non-ATP competitive type-III MEK1 inhibitors

Next, we asked what cellular mechanism may underlie the 6-OH exposure-induced attenuation of neuronal activity. Because we have previously seen 1) 6-OH exposure inhibit transcription of IEG *Arc* (Poston *et al.* 2018), and 2) MAPK signal transduction is required for *Arc* and other neuronal IEGs (Tyssowski *et al.* 2018), we wondered if 6-OH exposure may disrupt MAPK signaling. While closely inspecting 6-OH structure, we discovered that 6-OH shares key structural features with a class of kinase inhibitors that are commercially marketed as MEK1 inhibitors, a central kinase of the MAPK

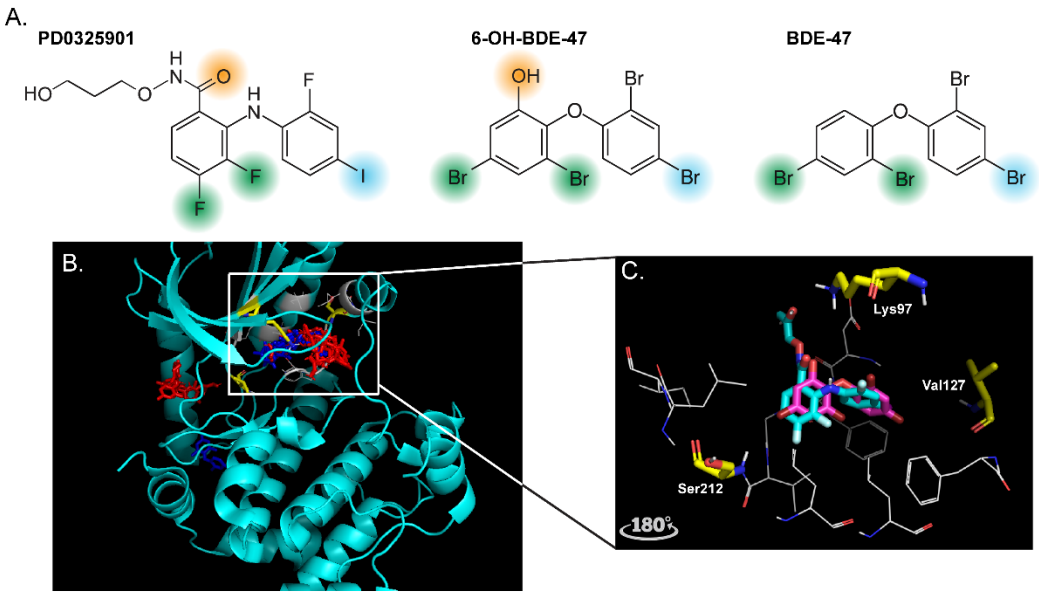


Figure 3-3. 6-OH-BDE-47 shares key structural features of non-ATP competitive type-III MEK1 inhibitors. (A) Chemical structures of the commercial MEK1 inhibitor PD0325901, BDE-47, and 6-OH-BDE-47 with key MEK-binding substituents highlighted. (B) Crystal structure of MEK1 (PDB: 3EQI) with top binding poses generated in ligand docking simulations for 6-OH and PD superimposed. (C) Rotated view of the allosteric binding pocket of MEK1 with most realistic generated poses of6-OH and PD.

pathway. It is thought that there are three key features that enable these specific small molecules to effectively inhibit MEK1, which are discussed at length elsewhere (Heald *et al.* 2012; Zhao *et al.* 2017b). Briefly, there are three major criteria: 1) a polar group that can engage the MEK1-Lys97, 2) a hydrogen bond acceptor to interact with MEK1-Ser212, and 3) a para-substituted ring opposite to the previous substituents that occupies a lipophilic pocket and interacts with MEK1-Val127– all of which 6-OH possesses **(Fig. 3A)**.

Given the importance of the MAPK pathway in neuronal activity-induced gene transcription (Tyssowski *et al.* 2018) and neuronal plasticity (Thomas and Huganir 2004), we hypothesized that the effects of 6-OH exposures discussed so far could be due to inhibition of the MAPK pathway. To test this hypothesis, and to gain insight into the plausibility of 6-OH binding to MEK1 as the small molecule inhibitor, we conducted Ligand-Protein docking simulations using published crystal structures of MEK1 and 6-OH as well as a commercial inhibitor, PD0325901 (PD). Utilizing AutoDock Vina (Trott and Olson 2010), which evaluated the best binding poses for each ligand, we found that both 6-OH and PD were placed around the well-characterized binding pocket for this type of inhibitor, without the software having been given any prior knowledge of the binding surface. Almost all of the generated top poses for both 6-OH and PD clustered around this surface when the simulation was initialized near the catalytic kinase domain **(Fig. 3B)**. The poses for 6-OH and PD that are closest to published binding modes of MEK1 inhibitors are superimposed in **Fig. 3C**. These results were encouraging and suggested that 6-OH is capable of interacting with the type of binding surface known to be targeted by small molecules specifically designed to inhibit MEK1.

3.4.3 Chronic MEK1 inhibition by PD attenuates synaptic activity

Informed by our *in silico* observations, we next asked if chronic PD treatment would mimic the effects of 6-OH chronic exposure on neuronal electrical activity (as in Fig. 1). We treated cortical neurons grown on MEAs with two mild doses of PD. As expected, both doses of PD significantly reduced spontaneous spiking across the recording period, which was quantified and is summarized in **Figure 3-S1**. This observation correlatively suggests that 6-OH may produce altered phenotypic outcomes – like, suppression of synaptic activity – via inhibition of the MAPK pathway. To test this hypothesis directly, we assessed the effects of acute 6-OH exposure in dissociated neurons in culture.

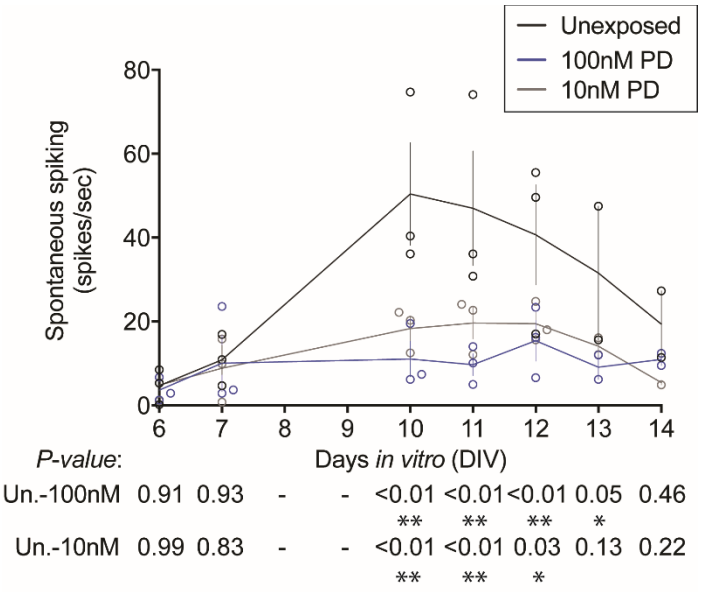


Figure 3-S1. Chronic PD0325901 exposure suppresses spontaneous neuronal activity. Primary embryonic rat cortical neurons grown on MEAs were chronically exposed to low doses of PD0325901, starting at the time of plating, in order to assess whether exposure to a commercial MEK inhibitor would produce similar effects as prolonged 6-OH-BDE-47 exposure. Detectable spontaneous activity was recorded and quantified across the first two weeks of growth in vitro, N=3. * indicates P-value <0.05, ** indicates P-value <0.01.

3.4.4 6-OH-BDE-47 inhibits MEK-ERK signaling in vitro

In mature neurons, MEK-ERK signaling conveys information from the membrane to many subcellular locations, including the synapse and the nucleus. To test the prediction that 6-OH is capable of directly inhibiting MEK1, and thus MEK-ERK signaling within cells, we acutely exposed primary cortical neurons to BDE-47 and its hydroxylated metabolites and then stimulated them with a treatment of Bic and 4AP. This treatment elevates intracellular calcium and induces the MEK-ERK signaling pathway, which may be read out as phosphorylation of ERK (pERK), the direct downstream target of MEK. We found that activity-induced cytosolic elevation of the pERK level was significantly reduced only in cells treated with 6-OH (**Fig. 4A**), but not the parent compound or other metabolites. While strong inhibition of pERK induction was observed with higher 6-OH concentrations, the lowest exposure level used (500nM) was ineffective. The 500nM dose, as we have previously estimated, is the approximate brain concentration reported from *in vivo* exposure studies of PBDEs in rodents (Poston *et al.* 2018) and is on the same order of magnitude as the EPA reported average for human exposure (U.S. Environmental Protection Agency (EPA) 2010). As we initially did not see a significant decrease in pERK levels with this environmentally-relevant concentration, we exposed cells to 500nM 6-OH for increasing amounts of time, yielding qualitatively mild but not significant inhibition in the whole cell extract (**Fig. 4B**). Considering subcellular location-specific functions of the MEK-ERK pathway, we next prepared nuclear extracts from neurons treated with 500nM 6-OH to observe only the amount of

pERK entering the nucleus (**Fig. 4C**). Again, we observed mild reduction in pERK levels after acute exposure that was not significant after quantification. At this point, we rationalized that 500nM 6-OH may be too weak to detectably inhibit strong pERK levels induced by the 50mM Bicuculline treatment. To titrate the stimulus strength, we activated neurons with 5mM Bicuculline without 4AP. With a weaker stimulus, we found that acute exposure to 500nM 6-OH significantly decreased nuclear levels of pERK (**Fig. 4D**). Taken together, 6-OH inhibits the MAPK pathway in neurons in a dose- and stimulus strength-dependent manner.

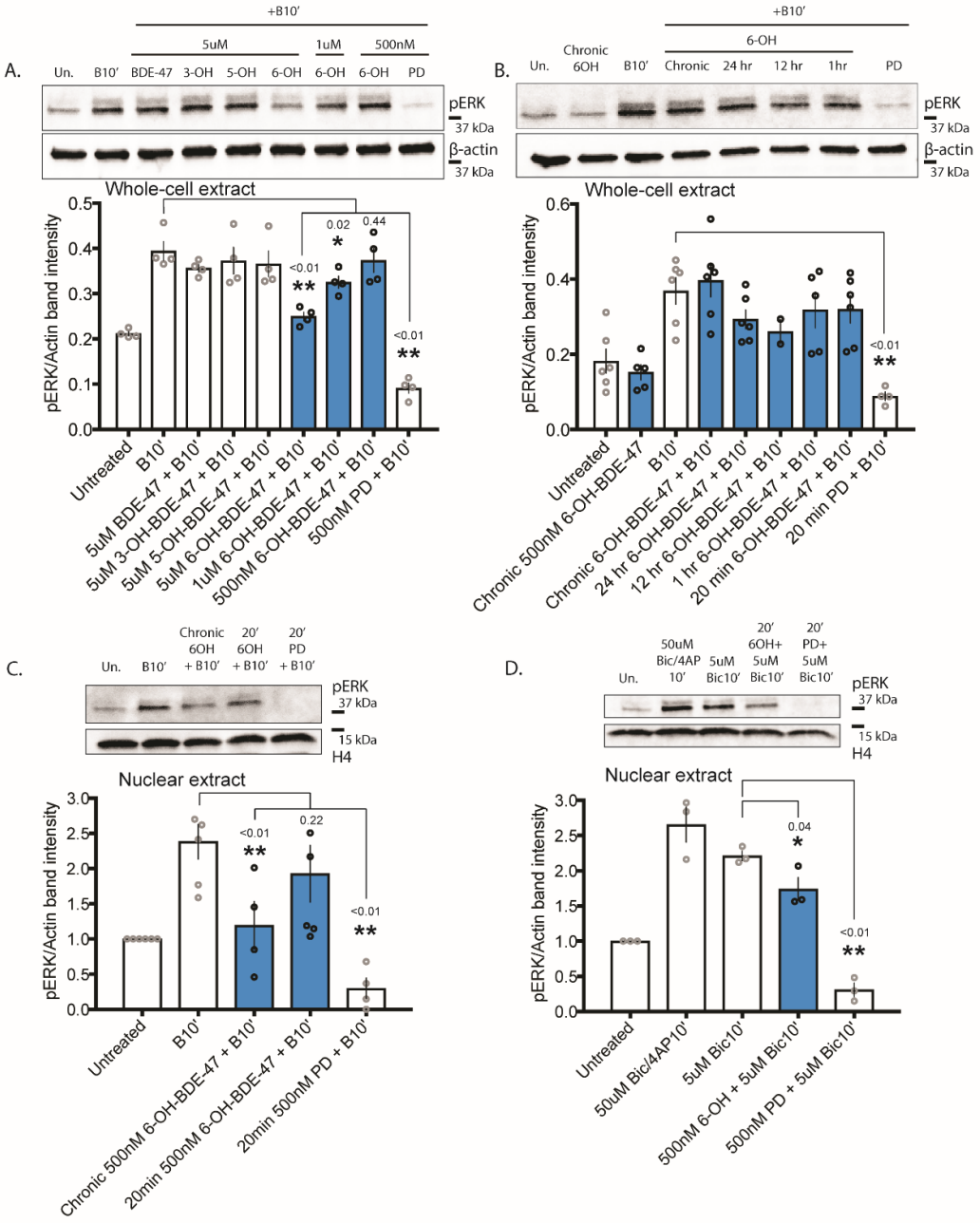


Figure 3-4. (previous page) Acute 6-OH-BDE-47 exposure inhibits activity-dependent pERK levels. Activity-induced MEK-ERK signaling was assessed by measuring pERK levels by western blotting. (A) Cells acutely exposed to BDE-47 or one of its hydroxylated metabolites were stimulated with Bic/4AP for 10 minutes before collecting whole-cell lysates, N=4. (B) Cells were acutely exposed to the lowest exposure dose, 500nM, for increasing lengths of time before Bic/4AP stimulation and whole-cell lysate collection, N=3-6. (C) To assess nuclear availability of pERK after 6-OH exposure, nuclear lysates were collected after10 minute Bic/4AP treatment, N=4-5. (D) Acute 6-OH exposed cells were treated with either Bic/4AP or a lower concentration of Bic by itself to assess the effect of stimulus strength on nuclear availability of pERK, N=3. * indicates P-value <0.05, ** indicates P-value <0.01.

Next, we measured pre-mRNA levels of the MAPK-dependent and neuron specific IEG *Arc* after acute PBDE exposure as a second approach to corroborate the above-mentioned pERK induction data. We acutely exposed neurons to BDE-47 and its hydroxylated metabolites as in Fig. 4A and found significant attenuation of *Arc* induction only after 6-OH exposure (**Fig. 5B**). To ensure that the observed effects were not solely caused by rapid feedback or effects at the synapse (on which the Bic/4AP treatment paradigm relies), we also stimulated cells with TTX and PMA. This treatment, which is previously described (Poston *et al.* 2018) and depicted in **Fig. 5A**, silences propagation of neuronal activity while activating MEK-ERK signaling likely through Protein Kinase-C (PKC). Again, we found that acute exposure only to 6-OH significantly attenuated *Arc* induction (**Fig. 5C**). Here the effect was not significant with a 500nM exposure, as with the cytosolic pERK data (**Fig. 4**), so we again examined whether the strength of the stimulus impacts the extent of inhibition by titrating the concentration of PMA the cells were treated with. As expected, we found that with a lighter stimulus, the extent of 6-OH-induced inhibition was greater, with *Arc* pre-mRNA levels significantly reduced when 6-OH exposed cells were stimulated with 0.1 and 0.01μM PMA (**Fig. 5D**). All together, these data suggest that the ortho-hydroxylated 6-OH, but not the parent compound or other metabolites, inhibits the MAPK pathway in neurons.

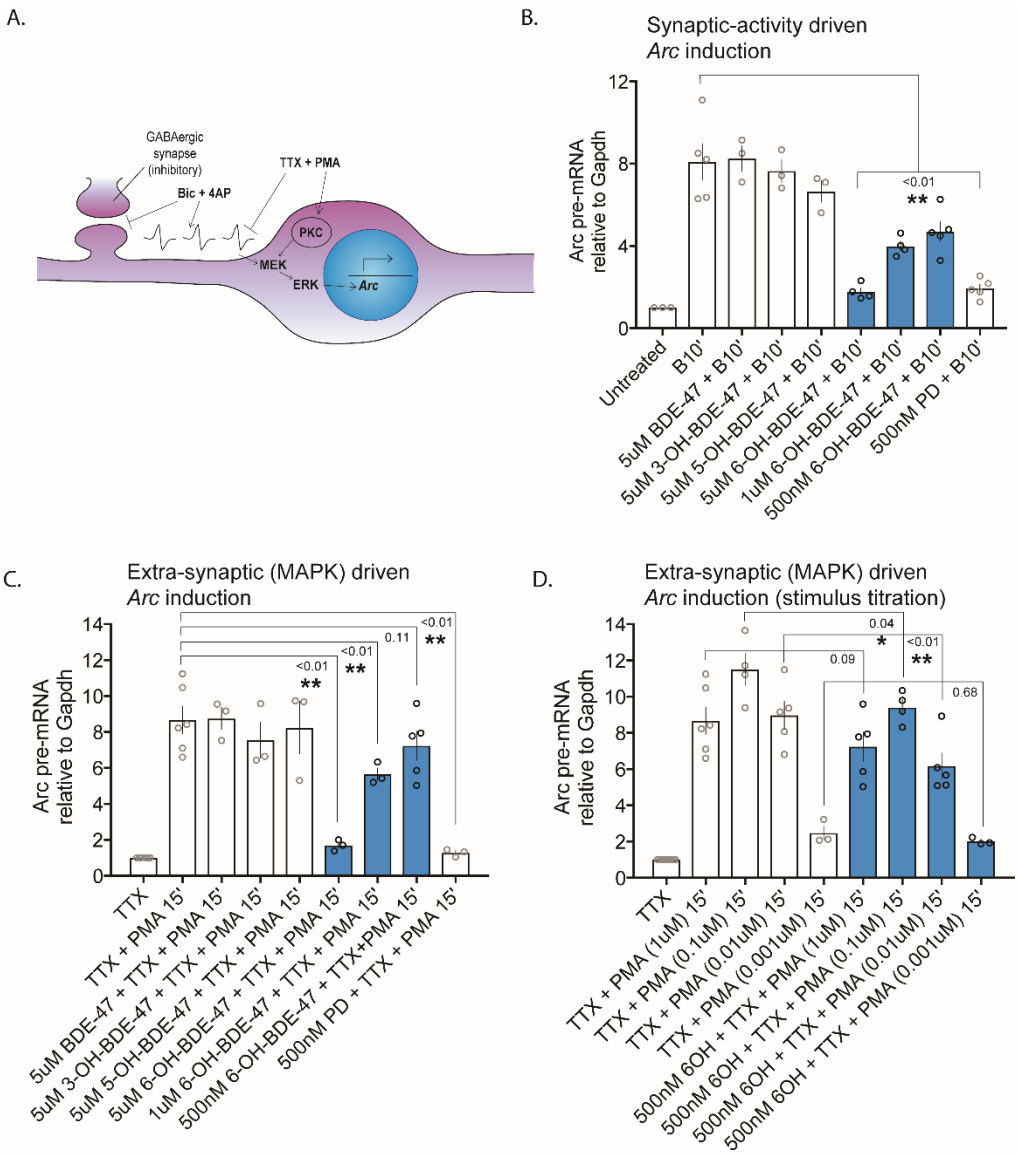


Figure 3-5. Acute 6-OH-BDE-47 exposure attenuates activity and MEK-ERK dependent *Arc* transcription. MAPK-dependent gene transcription was evaluated as a measure of 6-OH mediated MEK-ERK inhibition by quantifying levels of *Arc* pre-mRNA following multiple stimulation approaches. (A) Graphical depiction of the stimulation methods used–Bic/4AP synaptically activates MEK-ERK signaling, while TTX/PMA suppresses neuronal activity while activating MEK via PKC. Both ultimately drive induction of *Arc* gene transcription. (B) *Arc* pre-mRNA levels were estimated following exposure to BDE-47 or one of its hydroxylated metabolites and treatment with Bic/4AP, N=3-5 (C) Similar to B, except that cells were stimulated with TTX/PMA, N=3-6. (D) To assess the effect of stimulus strength on inhibition of *Arc* induction by 6-OH. cells were acutely exposed to 500nM 6-OH before stimulus with a range of PMA concentrations, N=3-6. * indicates P-value <0.05, ** indicates P-value <0.01.

3.4.5 Specific ortho-hydroxylated PBDE metabolites inhibit MEK-ERK signaling in vitro

To further test the generality of our hypothesis regarding how hydroxylated PBDEs like 6-OH can inhibit MEK-ERK signaling, we acutely exposed cells to several other ortho-hydroxylated PBDE metabolites, whose possession or lack of the substituents thought to be critical for MEK inhibition are depicted in **Fig. 6A.** These include BDE-99, the second most environmentally prevalent PBDE humans are exposed to (U.S. Environmental Protection Agency (EPA) 2010). It was interesting to find that only one of these ortho-hydroxylates metabolites – 6-OH-BDE-99 – reduced pERK induction following Bic treatment (**Fig. 6B**). This metabolite produced nearly an identical effect on *Arc* induction, at various concentrations, as 6-OH-BDE-47 (**Fig. 5B & 6C**). Together, our data suggests that for efficient MEK inhibition, ortho-hydroxylated PBDE metabolites require a para-substituted halogen on the same ring as the hydroxyl group (**Fig. 6A**), thus explaining why some ortho-hydroxylated metabolites and not others inhibit MEK and subsequent *Arc* transcription.

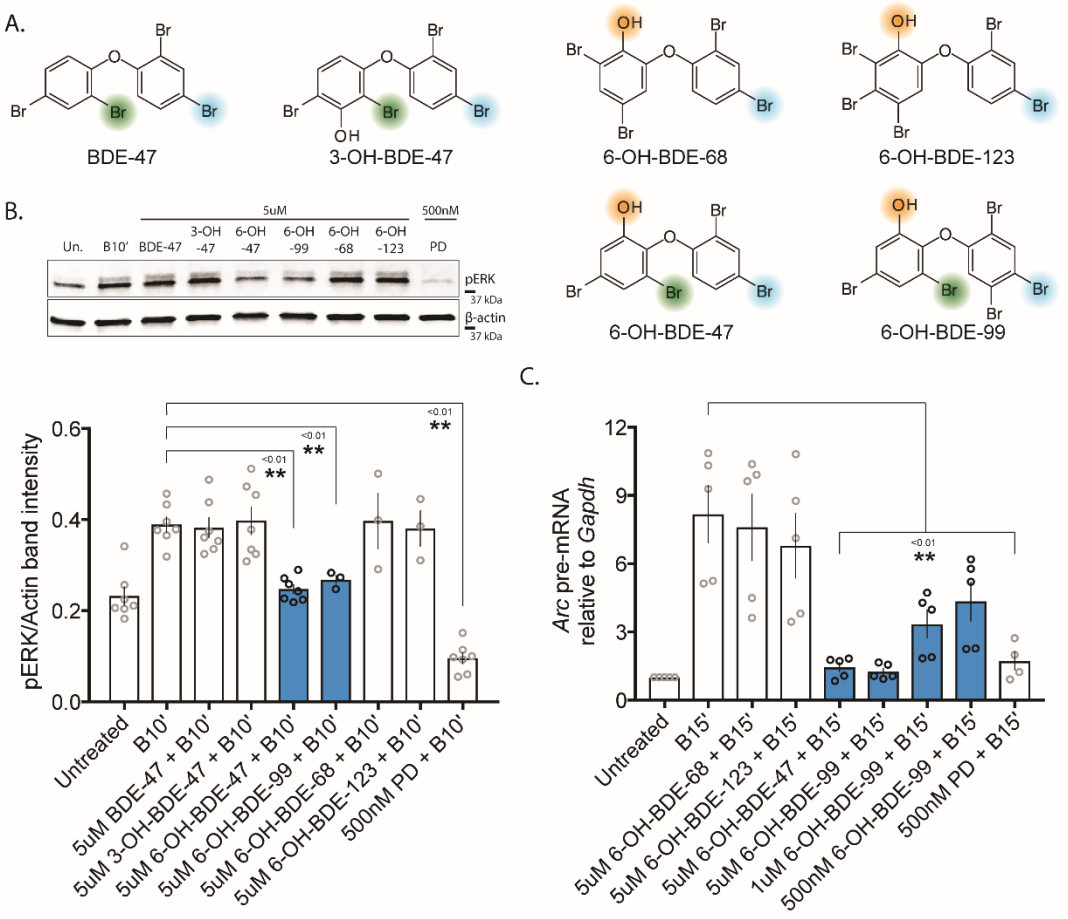


Figure 3-6. (previous page) Specific ortho-hydroxylated PBDE metabolites have the capacity to inhibit neuronal MEK-ERK signaling. In an effort to generalize our hypothesis that ortho-hydroxylated PBDE metabolites can inhibit MEK-ERK signaling, both approaches used to evaluate MEK inhibition by BDE-47 and its metabolites (Fig 4 and 5) were re-employed with several additional ortho-hydroxylated metabolites. (A) Chemical structures of PBDEs screened, with key substituents for potential MEK1 binding highlighted, specifically indicating the newly identified ortho-halogen thought to be critical for inhibition of MEK-ERK signaling. (B) Representative western blot measuring pERK levels after exposure to various PBDEs (top) with quantification of blots summarized (bottom), N=3 (C) Similar PBDE exposures as in B. but evaluating *Arc* pre-mRNA level attenuation by BDE-47 and BDE-99 metabolites, N=5. * indicates P-value <0.05, ** indicates P-value <0.01.

3.4.6 Axonal guidance is dysregulated in vivo by both 6-OH-BDE-47 and the MEK inhibitor PD0325901

Many developmental and functional processes in neurons are regulated by MAPK signaling (Samuels *et al.* 2009; Xing *et al.* 2016; Lyons and West 2011). There is some evidence that MAPK signaling is involved in axonal guidance. One study reported that a let-60c mutation (a *C. elegans* ortholog of KRAS, a membrane GTPase upstream of MEK) led to axonal guidance defects manifested as aberrant ventral midline crossing (Bülow *et al.* 2004). Another confirmed that MEK-ERK signaling regulates netrin-1 dependent axonal branching in cultured hamster sensorimotor cortical neurons (Tang and Kalil 2005). It has also previously been reported that primary hippocampal neurons exposed to BDE-47 and BDE-49 (and their hydroxylated metabolites) exhibited reduced axonal growth *in vitro* (Chen *et al.* 2017). To test the effects of 6-OH exposure *in vivo* in the context of MAPK inhibition, we exposed adult flies (*D. Melanogaster*) and their offspring to BDE-47, two of its hydroxylated metabolites, and PD. Then we assessed axonal guidance in the brains of offspring by observing effects on mushroom body ß-lobe axonal midline crossing. It may be noted that the amino acid sequence of MEK1 is highly conserved between *H. Sapiens*, *R. Norvegicus*, and *D. melanogaster* — including the three key residues known to be involved in binding non-ATP competitive type-III inhibitors (**Fig. 7A**). In wild type *Drosophila*, ß-lobe axons do not or rarely cross the midline. However, under conditions that encourage abnormal axonal growth, these axons cross the midline to various degree (mild, moderate and severe). The qualitative scheme for assessing the extent of midline crossing is depicted in **Fig. 7B** and the frequency of the different effect severities are summarized in **Fig. 7C**. We found that 6-OH and PD exposure produced qualitatively similar increases in midline crossing, with more mild effects from BDE-47 and 5-OH-BDE-47 (5-OH) exposures. This became more evident when the distribution of crossing frequencies was rank scored for each exposure and assessed quantitatively (**Fig. 7D**). Analysis of rank score revealed that 6-OH and PD shift the median effect from ‘none’ to ‘mild’ while the parent compound and 5-OH did not produce a significant change. These data indicate that 6-OH exposure can

dysregulate axonal guidance *in vivo* and suggest that the effect may be mediated via inhibition of developmental MAPK signaling.

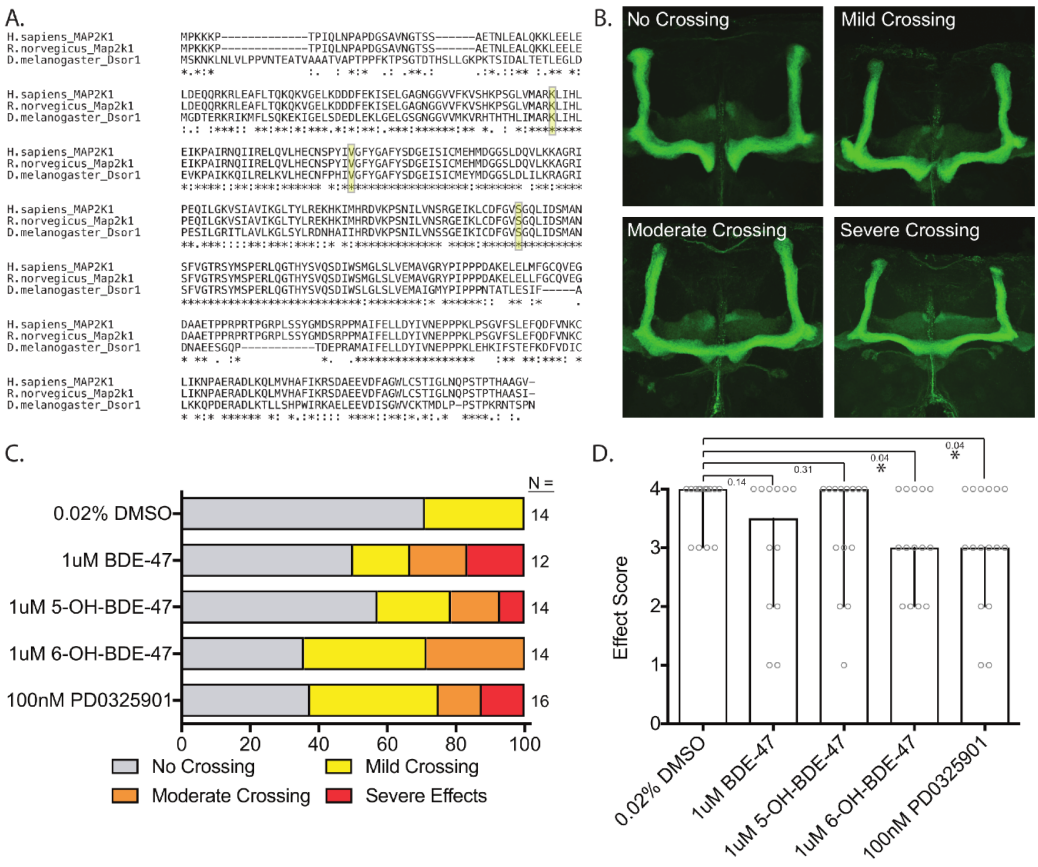


Figure 3-7. In vivo exposure to 6-OH-BDE-47 and the MEK inhibitor PD0325901 disrupt axonal guidance. Canton-S flies (D. melanogaster) were exposed to BDE-47, 5-OH, 6-OH, or PD via feeding. The mushroom bodies of offspring were then assessed for aberrant midline crossing. (A) Alignment of the amino acid sequences of human Map2k1 (MEK1) and its rat and fly orthologs. The proteins are highly conserved, including the three key residues thought to mediate allosteric MEK1 inhibition. (B) Example images of the effect categories used in the qualitative scoring scheme. (C) Crossing frequency by severity level for each of the treatment groups, N=12-16. (D) Crossing severity was rank scored as follows: No crossing: 4, mild crossing: 3, moderate crossing: 2, severe crossing and/or lobe malformation: 1. The median score of each treatment group is displayed after applying a numerical scoring scheme. P-values generated using the Mann-Whitney U test was used to compare the median of each group with that of the DMSO control, N=12-16. * indicates P-value <0.05.

3.5 Discussion

PBDEs are well known environmentally pervasive toxins that are concerning for human health, especially considering the growing body of evidence linking their exposure levels to behavioral abnormalities related to NDDs. Much work has been done characterizing the effects and mechanisms of toxicity of PBDEs, but there is still not a clear general understanding of how these compounds compromise the developing nervous system and how the effects are related to adverse phenotypic outcomes. In this chapter, I propose a novel mechanistic hypothesis regarding how certain hydroxylated metabolites of common PBDEs (including BDE-47 and BDE-99) exert effects in neurons. Here, we corroborate earlier findings indicating that exposures to an ortho-hydroxylated BDE-47 metabolite, 6-OH, impacts synaptic functionality and extended those findings by showing that 6-OH exposure affects neurodevelopmental processes *in vivo*. We also, for the first time, demonstrate that specific ortho-hydroxylated metabolites can inhibit MEK-ERK signaling directly.

From our previously published work, we had made two relevant predictions related to the effects of chronic 6-OH exposure: 1) effects in developing neurons likely manifest at the level of synaptic functionality and 2) 6-OH may be capable of inhibiting signaling at or downstream of PKC (Poston *et al.* 2018). Primary cortical neuronal cultures grown on MEAs (**Fig. 1&2**) allowed us to directly test the first hypothesis. While some recent studies have begun to employ MEAs to study PBDE developmental neurotoxicity as part of larger screens (Brown *et al.* 2016; Frank *et al.* 2017; Shafer *et al.* 2019), the data presented here are, to our knowledge, the first example of such evidence for hydroxylated PBDE metabolite-induced effects. They demonstrate that chronic nanomolar 6-OH exposures suppress both spontaneous and evoked electrical activity and that the exposures specifically appear to impact pre-synaptic composition. Interestingly, 6-OH was found to have a pronounced effect on evoked activity under a Bic washout treatment paradigm, which has been previously published as a model of *in vitro* plasticity (Arnold *et al.* 2005). This may partially explain the reported relationship between learning deficits and BDE-47/99 exposure in animal studies (Dorman *et al.* 2018), as it is known that their hydroxylated metabolites can be produced endogenously, primarily via CYP2B6, which is known to be dynamically expressed in the brain (Malmberg *et al.* 2005; Lupton *et al.* 2009; Miksys and Tyndale 2004; Feo *et al.* 2013a; Erratico *et al.* 2013; Fu *et al.* 2016).

In depth investigation of the second prediction generated from our previous work was spurred on by the serendipitous observation that 6-OH shares key chemical features with a class of inhibitors designed for MEK1, a central kinase in the MAPK signaling cascade (Plotnikov *et al.* 2011; Lyons and West 2011). These inhibitors target an allosteric binding surface on MEK1, which has been well characterized due to interest in these compounds as drugs for cancer

therapy (Price 2008; Cheng and Tian 2017). The structural similarities and results from Ligand-Protein docking simulations encouraged us to pursue 6-OH as a potential MEK1 inhibitor (**Fig. 3**). Using multiple approaches to activate MEK-ERK signaling, mediated both synaptically and pharmacologically (**Fig. 5A**), we found that acute 6-OH exposures inhibit induction of pERK levels and MAPK-regulated gene transcription (**Fig. 4&5**). Strong inhibition was found to be achieved at concentrations an order of magnitude higher than required with the commercial MEK inhibitor PD. This is consistent with the structure of 6-OH relative to PD, where 6-OH has a more minimal polar group that prevents additional interactions with MEK-bound ATP that further stabilize binding in the case of PD. Additionally, we found that time-of-exposure does not appear to increase the extent of inhibition by low concentrations of 6-OH (**Fig. 4B**) and that the strength of the stimulus driving MEK-ERK activation affects the extent of 6-OH-mediated inhibition, with lighter stimuli leading to stronger inhibition for both the synaptic and PMA-driven MEK activation (**Fig. 4D&5D**). Overall, it is likely that environmentally relevant levels of MEK-inhibiting ortho-hydroxylated metabolites are not sufficient to widely suppress MAPK signaling throughout the cell (especially given the extensive negative feedback opposing perturbations), but they may regionally accumulate to high enough concentrations to inhibit MEK in specific subcellular locations, an interesting possibility that is further discussed below.

In an effort to generalize our findings, we screened several other ortho-hydroxylated metabolites of BDEs-68/99/123 and found that the metabolite of BDE-99 could also inhibit MEK-ERK signaling when used to acutely treat cells (**Fig. 6**). While initially surprising, these data indicate that in order for an ortho-hydroxylated PBDE metabolite to be effective at inhibiting MEK-ERK signaling, it also specifically requires an ortho-substituted halogen that presumably interacts with MEK1 Ser212. Taken together, these several lines of molecular evidence demonstrate that specific ortho-hydroxylated PBDE metabolites can inhibit MEK-ERK signaling *in vitro*. This potential for acute MEK-ERK inhibition, combined with chronic-exposure-induced deficits in synaptic activity and composition, corroborates and further explains our previous findings concerning the ability of cortical neurons to induce *Arc* expression following chronic 6-OH exposure (Poston *et al.* 2018).

To validate our *in silico* and *in vitro* findings *in vivo*, we compared the exposure effects of PD to 6-OH using a neurodevelopmentally-relevant readout in fruit flies (*D. melanogaster*). *Drosophila* was chosen due to its relatively simplicity as an animal model and, importantly, the *Drosophila* ortholog of MEK1, *Dsor1*, is highly conserved– including the key amino acid residues that mediate allosteric inhibition (**Fig. 7A**). Further, it is known that MAPK signaling plays a role in developmental axon growth (Zhou and Snider 2006; Tang and Kalil 2005) and exposure to PBDEs have previously been reported to impact axonal growth *in vitro*(Chen *et al.* 2017). In our studies, exposure was accomplished via oral

administration to the P1 generation—to ensure embryonic exposure—and also to F1 larva to cover the neurodevelopmental period of the mushroom body formation. Adult offspring brains were examined immediately post-eclosion to assess the extent of mushroom body ß-lobe axonal midline crossing. Here, we found that 6-OH and PD exposures produced qualitatively similar increases in midline crossing, with BDE-47 and 5-OH also increasing crossing frequency, but to a lesser and insignificant extent (**Fig. 7C**). After applying a numerical scoring scheme and quantifying the effects, we found that 6-OH and PD shift the median effect to 'mild' while the parent compound and 5-OH did not produce a significant change. These data represent an *in vivo* confirmation of the effects of 6-OH exposure on axonal guidance and indicate that the effect may be mediated by MAPK signaling.

While these findings strongly indicate for an inhibitory potential of these metabolites, we note some nuanced aspects of the observed effects. For example, cultures chronically exposed to 500nM 6-OH were able to synaptically induce cytosolic pERK to the same extent as untreated controls, while significantly less pERK was available in the nucleus (**Fig. 4B&C**). This was unexpected, especially given the compromised electrical activity observed for chronically treated cells, but could be explained by the extensive homeostatic feedback mechanisms known to be employed by the MAPK pathway (Lake *et al.* 2016). Such feedback mechanisms may also explain the discrepancy between our findings and those of another recent report which showed that exposures to 6-OH selectively inhibited ERK5 phosphorylation, but not ERK1/2, in adult neural stem cells (aNSCs) (Li *et al.* 2013). In this study the authors incubate aNSCs overnight with 5μM 6-OH, followed by a stimulus with EGF/bFGF to induced MAPK, and report no decrease in the amount of pERK1/2. In addition to the data from the present chapter showing that after prolonged treatment with 6-OH, cortical neurons induce the same level of pERK as untreated controls, it has been shown in mouse embryonic stem cells (ESCs) that acute PD treatment reduces pERK levels, but between 12 and 24 hours later, pERK signal strongly returns (Chen *et al.* 2015). Therefore, it is possible that either feedback within the MAPK pathway or differences in signaling dynamics in aNSCs account for this discrepancy between our observation and that by Li and colleagues.

Interestingly, chronic 6-OH treatment-induced differences in cytosolic and nuclear pERK inhibition also suggest another nuanced aspect of PBDE toxicity: their sub-cellular localization and localized effects. Due to the highly lipophilic nature of PBDEs, they are likely to be more prominent within close proximity of membranes. Supporting this notion, it has been demonstrated that several PBDEs localize in higher amounts in mitochondrial and microsomal fractions collected from cerebral granule neurons (CGNs) (Huang *et al.* 2009). Accordingly, it will be interesting and necessary to evaluate the potential of ortho-hydroxylated PBDE metabolites to inhibit specific pools of MEK, which is also known to be localized in different cellular compartments, including the cytosol,

mitochondria, outgrowing neurites and synapses (Wortzel and Seger 2011; Mao and Wang 2016). Considering the effects of such compartmentalized MEK inhibition may help to generally explain some of the various known effects of exposure to certain PBDEs, especially well-documented mitochondrial toxicity (Costa *et al.* 2014). Further, mitochondrial toxicity may link PBDE exposure to NDD etiology given the known comorbidity of mitochondrial deficits and autism (Wong and Giulivi 2016). Compartmentalized MEK inhibition could also be involved in the strong effect seen on Synapsin-I (**Fig. 1C**), as it and other synaptic proteins are known targets of synapse localized MAPKs (Yang *et al.* 2017; Jovanovic *et al.* 1996). MAPK-Synapsin signaling is also known to play a role in pre-synaptic plasticity (Vara *et al.* 2009; Kushner *et al.* 2005; Cesca *et al.* 2010; Giachello *et al.* 2010), which may be related to the effects of chronic 6-OH exposure on neuronal activity reported here (**Fig. 1&2**), though more work is needed for confirmation.

Taken together, the findings presented here provide new evidence on the effects of exposure to hydroxylated PBDE metabolites and establish novel insight into the underlying molecular mechanisms that mediate their toxicity. The indication that PBDE metabolites are capable of inhibiting MEK-ERK signaling sets up several potential avenues for further investigation. One takes into account another aspect of our previously published work (Poston *et al.* 2018), that 6-OH exposure dysregulates expression of BAF chromatin remodeling subunits. This evidence, along with a recent preprint report concerning ERK1/2 regulation of neurodevelopmental Polycomb Repressive Complex (PRC) chromatin remodeling that further speculates about additional regulation of BAF complex composition (Semprich *et al.* 2019), suggests that 6-OH exposure may interact with chromatin remodeling via modulation of MAPK signaling. More closely related to the data presented here, it will be critical to investigate the potential links between ortho-hydroxylated PBDE inhibition of MEK-ERK signaling and the many established routes of PBDE toxicity, especially related to mitochondrial function and regulation of synaptic proteins. Overall, such future studies may help to clarify the growing evidence linking human PBDE exposures to NDDs and perhaps provide useful insight into these debilitating disorders.

Chapter 4: Discussion and future directions– Theory of developmental PBDE exposures and subcellular disruption of membrane bound structures as a general mechanism underlying toxicity of hydroxylated PBDE metabolites

The body of work presented here has generated several novel findings regarding the toxicity of hydroxylated PBDE metabolites. Together, these findings support an intriguing model with which to generate and test further hypotheses regarding the general mechanisms of PBDE toxicity, the effects of these cellular disruptions in the nervous system, and the relationship of those effects to neurodevelopmental disorders. In this chapter, I will reiterate relevant characteristics and human exposure details of PBDEs, lay out both specific mechanistic details and a general working model of developmental PBDE exposures, and discuss new hypotheses generated considering this model and the novel findings presented in earlier chapters.

Polybrominated diphenyl ethers are a class of organic small molecules with natural and anthropogenic sources. They were first patented for use as flame retardants in the 1960s and became heavily used in consumer products in the 1970s following introduction of legislation regarding fire safety, namely California's Technical Bulletin 117 (TB117). The widespread use of PBDEs and that they are not chemically bound in consumer products has allowed them to leach into the environment, where they are now persistently stable and detectable around the globe. They also are known to accumulate in households, primarily in dust, leading to human exposures, particularly for infants and toddlers, who tend to have a higher body burden than adults (U.S. Environmental Protection Agency (EPA) 2010). In the human body, the lipophilicity of PBDEs leads to accumulation in lipid membranes, particularly in adipose tissue and the brain. Transport into the brain is likely predominantly mediated by passive diffusion at multiple blood-brain interfaces, but may also be facilitated by the OATP family of thyroid hormone transporters (Pacyniak *et al.* 2010), allowing for facilitated crossing of the blood brain barrier (BBB) into astrocytes and the interstitial fluid and potential further trafficking into other neural cells (Schroeder and Privalsky 2014). Developmental exposures of the brain to PBDEs have been demonstrated to lead to defects in learning and memory in non-human animal studies and there is a growing body of evidence correlating exposure levels in humans to behavioral deficits related to NDDs (details and references in Chapter 1). Accordingly, a large body of research has investigated the mechanisms of PBDE neurotoxicity, identifying several major mechanisms including: dysregulation of thyroid hormone signaling, interference with Ca^{2+} homeostasis, disruption of mitochondrial function leading to excess ROS and apoptosis, as

well as alterations to epigenetic regulation (Costa *et al.* 2014; Poston and Saha 2019). Despite the progress that has been made, a clear understanding of the relationship between developmental PBDE exposures and observed behavioral deficits has not been achieved. Here I will present my working theory of developmental PBDE exposures and how their specific molecular and cellular effects may be related to NDDs, specifically integrating the findings generated from the experiments constituting the body of this thesis.

4.1 Routes of neurodevelopmental PBDE exposures: barriers to the brain

To understand the effects of PBDE exposure in the developing brain, a logical starting point is to first consider how and where these small organic molecules enter the brain. The theory and data on the topic of transport of various ions and molecules into the brain come from a variety of fields: early

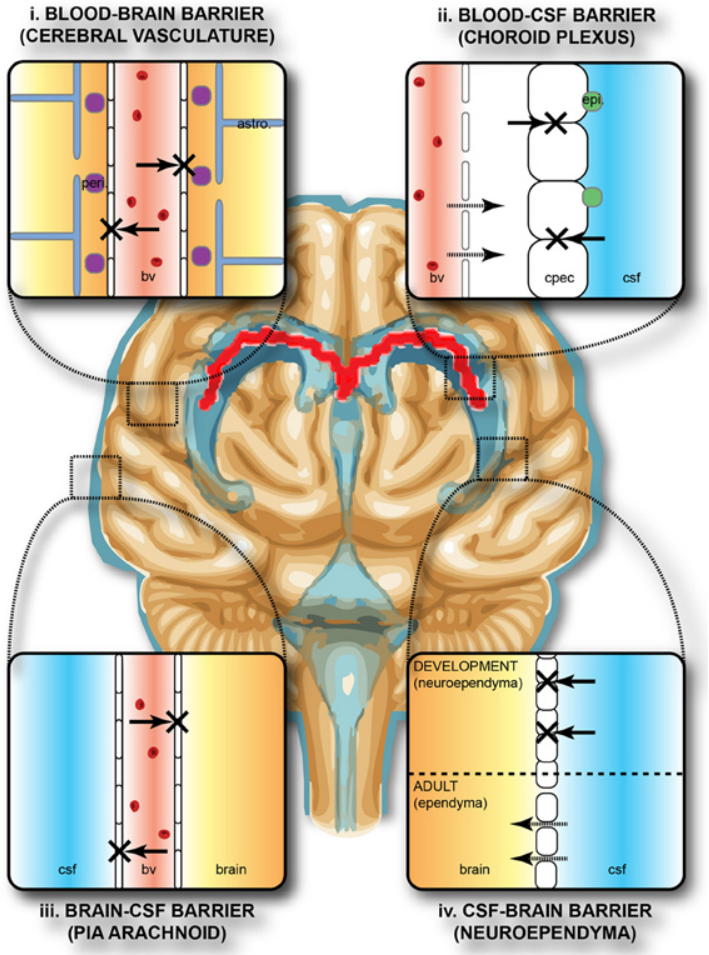


Figure 4-1. The protective barriers in the brain.

Abbreviations: astro, astrocyte; bv, blood vessel; cpec, choroid plexus; csf, cerebrospinalfluid; peri, pericytes. From Stolp *et al*. 2013, used under Creative Commons license

physiological work characterizing the tissues of the nervous system, neurotoxicological studies concerned with the vulnerability of the developing nervous system, and also from research conducted in the context of the delivery of therapeutic drugs in the central nervous system, which may be a particularly useful framework for informing hypotheses on neurodevelopmental exposures to PBDEs and other persistent organic pollutants. From this wide body of work, it has been established that there are four main barriers in the developing brain: the blood-brain barrier (BBB, the interface between neural cells of the brain and the cerebral blood vessels), the blood-CSF barrier (BCSFB, the interface between blood vessels and the cerebral spinal fluid formed in the choroid plexus of each ventricle), the brain-CSF barrier (the meningeal barrier between the brain and the CSF in the subarachnoid space), and the fetal CSF-brain barrier (a temporary barrier in the fetal brain preventing free diffusion from the ventricular CSF to the brain) (**Figure 4-1,** from (Stolp *et al.* 2013)). These interfaces between blood, CSF, and brain allow tight control of the influx and efflux of ions, nutrients, proteins, etc., providing a highly regulated and protected environment in the brain. At each of the barriers, there are cellular structures of varying complexity, but all rely on a layer of epithelial cells with tight-junctions that prohibit free diffusion in the space between the cells, restricting transport to mechanisms involving movement across the surrounding cell membranes. Uniquely, at the BBB, the endothelial cells surrounding the cerebral vasculature are additionally wrapped in astrocytic processes called 'astrocytic end-feet', collectively referred to as the 'neurovascular unit.' The critical properties of these barriers are established early in development, despite the common notion that the fetal BBB is 'immature' (an idea that has persisted in the face of much evidence to the contrary (Ek *et al.* 2012; Johansson *et al.* 2008)). Additionally, the developing brain is further shielded by a barrier between the maternal and fetal bloodstream comprised of a layer of trophoblast cells connected by tight-junctions, resembling the anatomy of the BCSFB at the choroid plexus (Metz *et al.* 1979).

4.2 Passive diffusion: facilitation and hinderance

Given the presence of the extensive barrier mechanisms in place protecting the developing brain, it would be natural to assume that it is relatively well protected from the influence of potentially toxic environmental insults. However, while these barriers are very efficient at regulating the transport of large macromolecules and even hydrophilic small molecules, it is much harder for them to exclude lipophilic small molecules (like PBDEs), whose movement across these barriers is theoretically limited only by their rate of diffusion across lipid membranes and re-partitioning back into the aqueous environment in cells or interstitial fluid. However, it is known that lipid diffusion of small molecules is limited by two key factors: their molecular weight (MW) and the number of hydrogen bonds they form. Typically it is held that the MW cutoff for efficient passive diffusion is in the range of 400 to 600 daltons (Da, 1:1 ratio with MW),

and that low numbers of hydrogen bonds facilitate lipid diffusion (Banks 2009; Pardridge 2012). A low degree of hydrogen bonding can also hinder the diffusion of small molecules, however, in that their partitioning back into aqueous milieus is deterred if they are too lipophilic. PBDEs (especially those with lower degrees of bromine substitution, tetra- and penta- congeners in particular) may possess an unfortunate balance with regard to these parameters, falling right within the 400-600 MW range, and forming in the range of 4-10 hydrogen bonds, depending on their metabolic state, with hydroxylation by monooxygenases providing an additional 2 hydrogen bonds. Such hydroxylation of xenobiotics is known to be mediated by cytochrome P450 (CYP) enzymes, which are expressed in the brain, particularly in microvessels, choroid plexus, and meningeal membranes (Ghersi-Egea *et al.* 1994), with CYP1B1 expression predominating in the microvessels (Dauchy *et al.* 2008). In fact, metabolism by CYPs is known to be a mechanism by which the BBB and BCSFB limit the passive diffusion of lipophilic small molecules by transforming them into typically less active forms that are more readily effluxed from barrier epithelial cells by transporters that will be detailed below (Strazielle and Ghersi-Egea 2013). Hydroxylation by CYPs would promote protective efflux of PBDEs diffusing into epithelial barrier cells, however, PBDEs are predominantly metabolized by CYP2B6 (Feo *et al.* 2013b), which has been detected in the brain (Gervot *et al.* 1999), but is only known to be expressed in neurons and astrocytes, including astrocytes surrounding cerebral blood vessels (Miksys *et al.* 2003). Thus, PBDEs may be capable of bypassing the protective mechanism of CYP-mediated metabolism at most of the various brain barriers and may be converted into hydroxylated metabolites with increased toxicity in astrocytes and neurons.

Transporters with a preference for metabolized substrates are not the only means of xenobiotic efflux. The barrier forming epithelial cells of the brain and placenta express a diverse class of membrane transporters known as ATP-binding cassette (ABC) efflux transporters. ABC-transporter expression is known to robustly restrict the entry of lipophilic xenobiotics in the adult brain, but while expression has been confirmed in the barrier cells of the developing and newborn brain, the functional degree of efflux efficiency has not been determined (Saunders *et al.* 2012; Ek *et al.* 2012). The main transporters involved in barrier forming cells are p-Glycoprotein (P-gp), which intercepts lipophilic molecules while they are crossing the cell membrane, and two others that primarily efflux intracellular metabolized substrates, breast cancer resistance protein (BRCP) and multidrug resistance related proteins (MRPs). Concerningly, there is evidence that PBDEs may not be good substrates for two of these predominant ABC-transporters, P-gp and BRCP. The study reporting these findings utilized artificial human vesicles with embedded P-gp and BRCP from which they could determine the internal concentration of BDE-47 and 6-OH-BDE-47 following exposure of the vesicles to the compounds (Marchitti *et al.* 2017). They found that the rate of uptake of the PBDEs was independent of the presence of ATP, which is necessary for the transporters to function. This suggests that both the

compounds are likely weak substrates for the transporters and that both are capable of appreciably crossing lipid membranes by passive diffusion. Another finding from the same and an additional study, show that BDE-47 and 6-OH-BDE-47 inhibit P-gp and BRCP activity, though only at relatively high concentrations (Nicklisch *et al.* 2016; Marchitti *et al.* 2017). Therefore, two of the main ABC-transporters preventing lipophilic small molecules from entering the brain (P-gp and BRCP) may be relatively ineffective in the case of unmodified and hydroxylated PBDEs (at least for BDE-47 and 6-OH-BDE-47), while BCRP- and MRP-mediated efflux of PBDEs may be avoided by the apparent lack of CYPs capable of mediating PBDE metabolism (though they would still be protective in the case of hydroxylated metabolites entering from the bloodstream). Given these data, and the well-documented vulnerability of the brain's barriers to small lipophilic molecules, it may be the case that PBDEs can relatively easily passively diffuse into the brain from the bloodstream at the interfaces described above (**Figure 4-1**).

While the lipophilicity of PBDEs may contribute to their ability to passively diffuse across the brain's restrictive epithelial cell barriers, it also limits the distance the compounds can efficiently spread by diffusion once in the cell-dense brain, which logarithmically decreases by a factor of approximately 10 for each 500μm from the point of entry (Fung *et al.* 1996). This indicates that PBDEs diffusing into the brain should tend to accumulate at the highest concentrations in cells surrounding points of contact with the CSF (i.e. cells proximal to the ventricles and other CSF-contacting surfaces of the brain, though for the latter, the CSF-brain barrier would have to be crossed) and with the cerebral vasculature (especially astrocytes contributing to the neurovascular unit). The number and distribution of cells thereby exposed to PBDEs would grow over the course of development, with choroid plexus and therefore CSF-proximal exposures highest at earlier stages of development relative to CSF-distal cells, depending on the ratio of CSF- to vasculature-exposed tissue (Saunders *et al.* 2012). With time, the increasing extent of vascularization and ventricular surface area would increase exposures, while the CSF concentration of PBDE and thus exposure level of ventricle-proximal cells would decrease as CSF volume increases so long as exposure levels of PBDE remained constant. The assumption that PBDE levels would remain constant across development is somewhat unrealistic, however, especially given the heightened exposure levels documented for young children, whose exposure level likely increases during early post-natal life due to higher rates of exposure to PBDEs in household dust and breastmilk (U.S. Environmental Protection Agency (EPA) 2010).

4.3 Active transport: thyroid hormone displacement

In addition to PBDEs entering the brain by passive diffusion, it appears that their crossing of the BBB can be facilitated by binding to the organic anion transporter polypeptide (OATP) family of thyroid hormone transporters given

several pieces of evidence. While thyroid hormones were long assumed to passively diffuse into the brain, they are now believed to be actively transported across the BBB by OATP transporters (Suzuki and Abe 2008). Recently, it has been demonstrated that several hepatic OATP transporters mediate PBDE uptake in liver, including OATP2B1 (Pacyniak *et al.* 2010), which has been found to be highly expressed in brain capillaries, suggesting a specific role in transporting thyroxine (T4) across the BBB (Gao *et al.* 2015). Given these data, it seems that PBDEs are likely actively transported across the BBB into astrocytes and the interstitial fluid and potentially further trafficked into other neural cells via additional transporters, potentially including neuron-localized OATPs (Schroeder and Privalsky 2014; Gao *et al.* 2015). The addition of active transport and trafficking through neural cells to the passive diffusion capabilities of PBDEs widens their potential reach in the brain and suggests that exposure level of cells surrounding the cerebral vasculature may be higher than would be expected from PBDE diffusion alone.

4.4 Summary of tissue level exposure

In summary, the model I propose here is that PBDEs enter the developing nervous system by a combination of passive diffusion and active transport, with the predominant sites of entry likely at points of CSF contact and via the cerebral vasculature. PBDEs that have not been metabolized are likely more capable of crossing the various brain barriers but are also potentially metabolized by CYP450s into more toxic hydroxylated metabolites in astrocytes and neurons. During pre-natal development, exposure would depend on the level of PBDEs in maternal blood and the extent to which they can cross the placental and developmental brain barriers. At earlier stages of neurodevelopment, crossing of PBDEs from the fetal blood into the CSF via choroid plexus would account for most of the exposure, while over time, increasing cerebral vascularization would lead to further exposure. After birth, the loss of the placental barrier, coupled with high rates of PBDE intake from dust and breastmilk may be responsible for the observed high PBDE levels. Ongoing neurodevelopmental processes during this period, such as axonal/dendritic outgrowth, synaptogenesis, and myelination may be at particular risk for disruption, though it is difficult to speculate on the relative vulnerability of the many critical processes at work across early brain development. With time and growth, subsiding exposure levels and a maturing brain likely lead to a reduction in PBDE-associated toxicity risk in the nervous system. This model realistically depends on additional parameters that are dynamic over the course of neurodevelopment, such as changing properties of the cells making up the various brain barriers (particularly expression of transporter proteins), the influence of the loss of placental protection at birth relative to the newborn's own blood filtering capabilities, rates of CSF clearance relative to PBDE influx, increasing blood volume relative to exposure level, etc. However, it provides a reasonable framework for generating hypotheses concerning neurodevelopmental PBDE exposures, given the likely mechanisms

and routes of PBDE entry. For example, based on this exposure model and considering the potential for some hydroxylated PBDE metabolites to inhibit MAPK signaling (see Chapter 3), I developed a series of experiments to test the effects of PBDEs to affect CSF-proximal cells in the developing brain. After injecting BDE-47 and 6-OH-BDE-47 into the lateral ventricles of embryonic rats, markers of neuronal differentiation can be monitored to detect effects on the pool of radial glial cells (RGCs, the progenitor cells from which cortical neurons are derived) surrounding the ventricles that would be most heavily exposed to PBDEs in the CSF. In particular, Tbr2, a marker of intermediate progenitor cells (differentiated RGCs migrating into the forming cortex) can be used given that it has been reported that pharmacological inhibition of MEK-ERK signaling in the developing brain led to an increased number of Tbr2 positive cells (Rhim *et al.* 2016). Markers for additional cell populations derived from exposed RGCs at the ventricles further allow for monitoring of potential PBDE-induced effects. Some example images are shown here (**Figure 4-2**), while quantitative results from initial experiments remain preliminary. At present it does not appear that there is a striking difference when several PBDEs were injected at a level of 500nM. This experiment illustrates, however, how hypotheses can be informed by both the developmental exposure model I describe here and known molecular effects of PBDEs. It will be interesting to further investigate the effects of PBDEs injected *in utero* on other neurodevelopmental processes (including axonal guidance, particularly in axon-dense regions close to the CSF, see results from Chapter 3) and CSF-proximal cells, especially given the control of exposure level and timing afforded by this approach. Studies aimed at testing the effects of PBDE exposure on cells near the cerebral vasculature can additionally be conducted, but would be more practically achieved utilizing either feeding of the compounds or injections outside of the brain preceding and throughout developmental stages at which cerebral vascularization is becoming prominent.

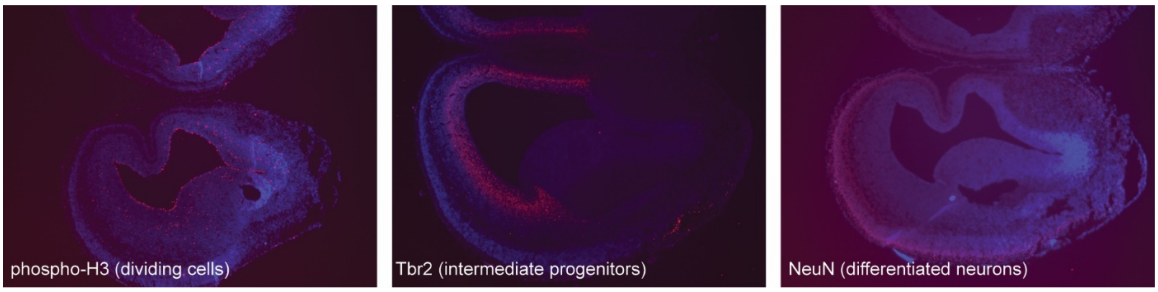


Figure 4-2. Monitoring the developing cortex *in vivo*. Coronal sections of embryonic day 18 rat brains. Example images are from unexposed controls from the *in utero* PBDE injection experiments described here. Markers of various cell populations relevant to cortical development are indicated. H3- histone 3; Tbr2- T-box brain protein 2 or eomesodermin; NeuN- Rbfox3 (originally referred to as antigen for “Neuronal Nuclei”)

4.5 Cellular PBDE exposure: the importance of sub-cellular distribution

In addition to the tissue-level exposure model detailed thus far, considering the cellular fates of PBDEs that make it into the brain is imperative for understanding their toxicity. The lipophilic nature of PBDEs that I have discussed as a major factor influencing their entrance into the brain likely also dictates how they behave in neural cells. PBDEs, once past the barriers of the brain, are free to diffuse throughout the interstitial fluid and into cells they come into contact with. They may also be actively trafficked in a manner similar to thyroid hormones. It is important to note here that it is thought that the presence of PBDEs in neural cells may exert either direct effects or indirectly influence exposed and surrounding cells by disrupting thyroid hormone homeostasis and signaling (Dingemans *et al.* 2011). In this body of work, I have largely focused on investigating direct, non-thyroid hormone related effects, and will continue that focus in the discussion here. By either diffusion or trafficking, once PBDEs come to reside in a neural cell, their lipophilicity should lead to their accumulation in the various membranes of these cells, including membrane bound organelles. This has been found to be the case in a study of PBDE accumulation in cerebellar granule neurons that reports the highest levels of accumulation of tested PBDEs in the microsomal (vesicles-like artifacts of cell lysis formed from membranes, largely from the endoplasmic reticulum) and mitochondrial fractions (Huang *et al.* 2010). Results from this study that have also been reported elsewhere highlight another important aspect of the kinetics of cellular PBDE accumulation– that PBDEs rapidly accumulate in cells and appear to magnify in concentration over time, sometimes to levels orders of magnitude higher than the concentration they are exposed to (Mundy *et al.* 2004; Kodavanti *et al.* 2005; Huang *et al.* 2009). This trend suggests that PBDEs may accumulate in exposed cells to higher concentrations than detected in the blood, perhaps even in a sub-cellular specific manner in and around membranes and membrane bound organelles. It also suggests that reports of brain concentrations of PBDEs from tissue may underestimate the levels that are actually present in specific sub-cellular locations, which could be modeled and directly tested going forward. Aside from the issue of the concentration of accumulating PBDEs in neural cells, the combination of available experimental observations and theoretical inferences regarding the intracellular distribution and kinetics of PBDEs presented here support the notion that PBDEs accumulate in a sub-cellular specific manner in and around membrane bound cellular structures. I believe this will be a critical concept for generating future hypotheses towards a more complete understanding of the effects of PBDEs in the developing nervous system and their relation to NDDs. With this in mind, I will close this chapter by briefly discussing the novel findings presented here within the context of the neurodevelopmental exposure and sub-cellular distribution models detailed above.

Taking into account the sub-cellular distribution of PBDEs and acknowledging their proclivity for accumulation in membrane bound structures may go a long way towards understanding some of the findings presented here and the known effects of PBDE exposures. Unmetabolized PBDEs entering the developing brain in the manner described in this chapter would most heavily accumulate in the membrane bound organelles of cells proximal to the CSF and to the cerebral vasculature. These would include astrocytes participating in neurovascular units and any cells close to the outer surfaces of brain regions bathed in CSF and proximal to the ventricles. Little is known about the effects of PBDEs in astrocytes– given that they may be some of the most directly and heavily exposed cells in the brain, future studies should explore potential effects in astrocytes. PBDEs accumulating in exposed neurons and astrocytes, especially in their mitochondria and endoplasmic reticuli, would be potentially subject to metabolism by CYP450 enzymes, producing hydroxylated metabolites with higher potential for toxicity. This process would be exacerbated by the high number and activity of mitochondria required to sustain the energetically demanding neurons. Thus, regions of high parent compound concentration may facilitate relatively high concentrations of hydroxylated PBDE metabolites.

4.6 New hypotheses and future directions

As shown in Chapter 3, certain hydroxylated metabolites seem to be capable of inhibiting MEK-ERK signaling. High levels of hydroxylated metabolites in neuronal mitochondria may therefore specifically disrupt MEK-ERK signaling in and around the organelles. This is an intriguing notion, as there is a growing appreciation for sub-cellular localization mediating specific MEK-ERK functions, and for the complex activation and trafficking of distinct pools of MAPKs between the cytosol, mitochondria, and nucleus (Alonso *et al.* 2004; Wortzel and Seger 2011). This complex trafficking has recently been shown to be sensitive to redox conditions including concentrations of hydrogen peroxide (Helfenberger *et al.* 2018). Reduced MEK-ERK signaling in neurons may further harm neuronal mitochondria by a mechanism involving activity-induced transcription of a MEK-ERK dependent gene, Npas4. Npas4 is an activity-induced transcription factor that has been shown to repress the mitochondrial calcium uniporter (Mcu) during periods of elevated activity, thus preventing Ca2+ overload and toxicity in mitochondria (Qiu *et al.* 2013). Taken together, these data indicate that PBDE-induced disruption of MEK-ERK signaling may be connected to the well-known effects of PBDEs on disruption of mitochondria and their redox processes. A natural continuation of the work presented in this dissertation would be to explore the potential connection between mitochondrial PBDEs, MEK-ERK signaling, and mitochondrial dysfunction, including isolating neuronal mitochondria for experiments involving exposure to hydroxylated PBDEs. Another future direction is to explore the potential accumulation of PBDEs in neuronal pre-synapses, which undergo a high rate of membrane turnover and contain a large number of membrane bound structures–synaptic vesicles. Data from Chapter 3 suggest that

pre-synapses and axonal guidance are affected by hydroxylated PBDEs. This may be due to the large number of vesicles and high rate of membrane turnover present in pre-synapses and axonal growth cones. A specific point of disruption may again be MEK-ERK signaling, particularly in regulating synapsin dynamics, a well-known MAPK regulated protein involved in pre-synaptic physiology (Cesca *et al.* 2010). Finally, PBDE-induced MEK-ERK and mitochondrial disruption may also be connected to the observed epigenetic effects of PBDE exposure. There is a growing body of evidence connecting mitochondrial dysfunction to alterations in nuclear DNA methylation (reviewed elsewhere (Minocherhomji *et al.* 2012))– the most extensively studied epigenetic disruption of PBDE exposure. Altered neuronal MEK-ERK signaling may also be related to the finding reported here in Chapter 2 concerning PBDE-exposure induced dysregulation of BAF complex subunit expression. Little is known about the transcriptional mechanisms regulating the expression of BAF complex subunits, and MAPK signaling is possibly involved. This idea is supported by a recent preprint report detailing the role of ERK signaling in the eviction of PRC complexes from neural genes (Semprich *et al.* 2019) and by the known role of the BAF complex in evicting PRC from heterochromatin (Kadoch *et al.* 2017). It appears that declining ERK signaling leads to the irreversible eviction of PRC complexes from neural genes, which I propose may be due to alterations in ERK-regulated BAF complex subunit expression that affects the ability of BAF to evict PRC complexes. Study of the roles of PBDE-disrupted MEK-ERK signaling in regulating such BAF subunit transcription is another future direction generated from the work presented here, and the last that I will discuss.

Going forward, I hope that this body of work will be of value in continuing to build an understanding of the effects of PBDE exposures in the developing nervous system, how these exposures are related to observed behavioral deficits in animals, generating insight regarding the interplay between genetic and environmental risk factors in neurodevelopmental disorders, and, generally, in appreciating the fascinating interconnected nature of molecular and cellular processes in the nervous system that act as the biological substrates of information processing underlying its ability to generate, perceive, and know.

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